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INFLUENCE OF HYDROLYSIS OF GLUCOSINOLATES ON THE NUTRITIVE VALUE OF RAPESEED MEALS

by

© IN KEE PAIK

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled INFLUENCE OF HYDROLYSIS OF GLUCOSINOLATES ON THE NUTRITIVE VALUE OF RAPESEED MEALS submitted by IN KEE PAIK in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in POULTRY NUTRITION.

ABSTRACT

A series of experiments were undertaken to study the hydrolysis products of rapeseed glucosinolates, their biological effects and the use of antidotes to counteract their deleterious effects.

In the first experiment (Section A) the products of enzymatic hydrolysis of rapeseed glucosinolates from a number of rapeseed varieties were determined. The results indicated that on autolysis of raw meals, the main hydrolysis products were nitriles. In addition to hydroxy--nitriles, two non-hydroxy epithionitriles (1-cyano-3,4--epithiobutane and 1-cyano-4,5-epithiopentane) were identified. They were present in all of the varieties tested (Candle, Torch, Regent, Midas and Tower) and were especially high in raw Torch meal. Goitrin was the predominant product from heat-treated meals regardless of source of the myrosinase. It was observed that the enzyme activity of semi-purified myrosinase from rapeseed was much lower than that of myrosinase from mustard seed.

In the second experiment (Section B) the effect of heat-treatment and conditions of enzyme hydrolysis of rapeseed meals (RSMs) on the concentration of nitriles and goitrin in the meals and the effect of treated RSMs on the performance of broilers were studied. An increase in incubation time, and freeze-drying after hydrolysis reduced total nitriles. A hydroxy nitrile, 1-cyano-2-hydroxy -3-butene, was least affected by freeze-drying. Inclusion in

the ration of 20% raw or heated Tower RSM hydrolyzed in different ways had little effect on rate of growth, feed efficiency, mortality or incidence of perosis but the size of the thyroid glands in the treated groups was 2 to 3 times larger than in the controls. Feeding 20% raw or heated Midas meal hydrolyzed in different ways depressed rate of growth and decreased feed efficiency. The growth depression was greater with the raw Midas meals containing or having potential to produce high levels of nitriles than with the heated meals containing or having potential to produce a high level of goitrin. The size of the thyroid glands was 4 to 20 times as large as those in the control group.

In the third experiment (Section C) the effects of feeding sodium thiosulfate or hydroxo-cobalamin (vitamin $B_{12}a$)on the performance of rats fed RSMs prepared to contain high levels of nitriles or goitrin were studied. The addition of 0.1% sodium thiosulfate to rations containing high levels of nitriles resulted in improved performance of rats but no antidotal effect of hydroxo-cobalamin was observed with meals of high-nitrile or high-goitrin content. The addition of sodium thiosulfate caused a decrease in size of kidneys and livers of rats in the high-nitrile groups which had larger kidneys and livers than the non-RSM control. Feeding diets containing either raw or heated Midas RSM resulted in a significant increase in serum thiocyanate content and excretion of thiocyanate in the urine. Addition of sodium thiosulfate resulted in a significantly higher

thiocyanate content in the serum of rats fed meal of high nitrile content. Thiocyanate excretion in the urine was lower when Midas meals were fed after being pre-hydrolyzed and freeze-dried than when meals were fed without hydrolysis.

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I. INTRODUCTION

Commercial production of rapeseed in Canada has increased rapidly since 1942 when the crop was introduced to produce a source of oil for use in lubricating marine engines. The crop was well adapted to growing conditions in Western Canada so acreage gradually increased until 1956-57 when the first edible rapeseed oil was extracted. Production in the 1956-57 crop year was 136,000 tons. Since that time the size of the crop has expanded quickly reaching 3.8 million tons in the 1978-79 crop year. Canada is now the largest producer and the largest exporter (1.7 million tons in 1978-79) of rapeseed in the world.

The availability of large supplies of rapeseed has resulted in the development of a fairly large oil processing industry. In 1979 domestic processing of rapeseed was 769,000 tons which resulted in the production of 314,000 tons of oil and 443,000 tons of rapeseed meal (RSM). Rapeseed oil now supplies approximately 44% of the oil used by Canada's edible oil industry. The meal produced as a by-product of oil extraction is used as a protein supplement in rations for livestock and poultry.

Although the amino acid composition of the protein of RSM is similar to other plant protein supplements such as soybean meal, some difficulties have been experienced when RSM is used in poultry rations. One of the main problems encountered has been related to the presence of glucosinolates in the meal. When the glucosinolates are

hydrolyzed they yield isothiocyanates, thiocyanates and nitriles, the concentrations of which will vary depending on the level of glucosinolates in the meal and the conditions of hydrolysis.

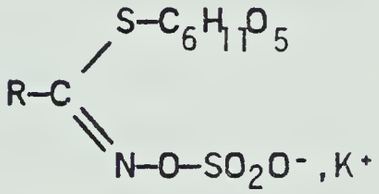
Since the presence of hydrolysis products of glucosinolates in RSM may exert harmful effects on poultry if their concentration in the ration fed is too high, experiments were undertaken to identify and determine the levels of hydrolysis products of glucosinolates in RSM produced experimentally from different varieties of rapeseed. In addition, the influence of the hydrolysis products on the performance of broiler chicks and rats was assessed.

II. LITERATURE REVIEW

A. Glucosinolates

General

Glucosinolates are found in the parenchymal tissues of plants, particularly those of the *Cruciferae* family (Kjaer 1960). Of a total of more than 75 individual glucosinolates identified (Kjaer 1978) more than 60 have been found in plants belonging to the *Cruciferae* family (Kjaer 1976). The name "glucosinolates" was suggested by Ettlinger and Dateo (1961) but the compounds have also been called mustard oil glucosides or thioglucosides. The general structure of the glucosinolates, which was definitely established for sinigrin (Ettlinger and Lundeen 1956), is shown in the following formula;



Different glucosinolates vary only in the structure of the side chain R in the formula. All glucosinolates are coupled with potassium cation except sinalbin which is a salt of sinapine. According to the nature of the side chain which is derived from amino acids, glucosinolates are divided into four major categories;

Group I. Aliphatic glucosinolates containg a *C*-methyl group or derived from diacids.

Group II. ω -methylthioalkylglucosinolates and

derivatives.

Group III. Arylmethylglucosinolates

Group IV. 2-arylethylglucosinolates and derivatives (Kjaer and Larsen 1973).

According to Kjaer (1978), pure glucosinolate salts are non-volatile, high-melting compounds, readily soluble in water by virture of their combined glucoside and salt character, and levo rotatory in aqueous solution (Kjaer 1978). The excellent crystallization properties of a few glucosinolates such as sinigrin, sinalbin and glucoiberin are in marked contrast to the generally experienced difficulties in inducing the other purified glucosinolates to crystallize. In a number of instances, acetylation of amorphous glucosinolates was advantageously employed for characterization purposes. Most glucosinolates and their acetates crystallize from water or aqueous alcohol as monohydrates (Kjaer 1960). Due to the character of substituted sulphates of glucosinolates, they can be quantitatively retained on anion exchange column, especially on anionotropic acid-washed alumina, and thus be freed from impurities such as sinapine and coloured complex. Subsequent elution with KOH or sulphate solutions afforded highly purified glucosinolates (Schultz and Gmelin 1954). Unlike glucosinolates, trimethylsilyl (TMS) derivatives are sufficiently volatile enabling GLC analysis of intact glucosinolates without hydrolysis (Underhill and Kirkland 1971; Persson 1974; Thies 1974, 1978).

Glucosinolates that have been identified in the seed of *Brassica campestris* and *Brassica napus* are presented in Table II.1 (reviewed by Josefsson 1970; Appelqvist 1972; Olsen and Sørensen 1980). Gluconapin, glucobrassicanapin and gluconasturtiin produce volatile isothiocyanates. Glucoraphanin and glucoalyssin are non-volatile isothiocyanate-producing glucosinolates which account for only 5% of the total intact glucosinolate content. Progoitrin and gluconapoliferin produce oxazolidinethione (OZT). Sinalbin which was detected in appreciable amounts in some of the rapeseed vatieties produces thiocyanate ion (Olsen and Sørensen 1980). The principal glucosinolates present in RSM are progoitrin, gluconapin and glucobrassicanapin (Josefsson 1970). The level of progoitrin was much higher in *B. napus* (4-12 mg of goitrin per g oil-free meal) than in *B. campestris* (0.5 mg of goitrin per g oil-free meal) while the level of gluconapin and glucobrassicanapin was almost same in both varieties (3.5-4 mg of isothiocyanate). The ratio of gluconapin to glucobrassicanapin shows difference, *B. napus* being relatively richer in the former and *B. campestris* having approximately equal amount of both glucosinolates (Downey et al. 1969; Bell and Jeffers 1976). Expressed as total intact glucosinolate, most of *B. napus* RSMs contained 5-6% glucosinolate while Bronowski RSM contained approximately 0.5% glucosinolate (Josefsson and Appelqvist 1968).

The biological role of glucosinolate in plant is not

Table II.1 Glucosinolates present in seeds of *B. campestris* and *B. napus*[1]

Trivial Name	Semi-systematic Name	Side-chain R
1. gluconapin	3-butenylglucosinolate	$CH_2=CH(CH_2)_2$
2. glucobrassicanapin	4-pentenylglucosinolate	$CH_2=CH(CH_2)_3$
3. glucoraphanin	4-methylsulphinylbutylglucosinolate	$CH_3SO(CH_2)_4$
4. glucoalyssin	5-methylsulphinylpentylglucosinolate	$CH_3SO(CH_2)_5$
5. gluconasturitiin	2-phenylethylglucosinolate	$C_6H_5(CH_2)_2$
6. progoitrin	2-hydroxy-3-butenylglucosinolate	$CH_2=CHCHOHCH_2$
7. gluconapoliferin[2]	2-hydroxy-4-pentenylglucosinolate	$CH_2=CHCH_2CHOHCH_2$
8. sinalbin[3]	*p*-hydroxybenzylglucosinolate	$(p)HOC_6H_4CH_2$

[1] From Josefsson (1970) and Appelqvist (1972)
[2] Name from Gronowitz et al. (1978)
[3] Olsen and Sørensen (1980)

well understood. Josefsson (1970) referred to gluosinolates as secondary natural products that appear as a result of metabolism, but that do not serve any essential function in the life of the individual plant. Kjaer (1978) suggested that since the sulphate group of glucosinolate is an excellent leaving group, the biological function of glucosinolates may be to serve as depositories of volatile reaction products, posessing specific biological properties. In fact, it was indicated that isothiocyanate, and perhaps other degradation products as well, play a decisive role in several well-established and specific parasite-host relations within important plants of the *Cruciferae* family.

Synthesis of glucosinolates

The biosynthetic studies have shown that major rapeseed glucosinolates, that is, 3-butenylglucosinolate, 2-hydroxy--3-butenylglucosinolate and 4-pentenylglucosinolate were synthesized from methionine *via* linear chain elongation and oxidative modifications (Chisholm and Wetter 1964, 1967; Serif and Schmotzer 1968). Detailed biosynthetic pathways were reviewed by several authors (Josefsson 1970; Underhill et al. 1973; Kjaer and Larsen 1973; Kjaer 1976, 1978). Homologous amino acids are derived from methionine by mutiple chain-elongation with the methyl carbon of acetate. After a series of enzymatic reaction, amino acids are transformed to aldoxims. Aldoxim incorporates sulphur leading to the formation of thiohydroximate. The most effective source of the thiohydroximate sulphur was

cysteine. Thiohydroximate and glucosyl transferred from UDP-glucose form desulphoglucosinolate by the action of UDP-glucose-thiohydroximate glucosyltransferase. The *N*-sulphate ester of glucosinolates is derived by the transfer of sulphate from 3′-phosphoadenosine--5′-sulphatophosphate to desulphoglucosinolate.

Some glucosinolates are derived from amino acids without chain-elongation; for example, conversion of tryptophan into indol-3-ylmethylglucosinolate (Kutáceк et al. 1962), phenylalanine into benzylglucosinolate (Underhill et al. 1962) and tyrosine into sinalbin (Kindl 1965).

It was shown that the hydroxyl group of hydroxy analogues of phenolic glucosinolates is introduced at the amino acid stage of biosynthesis (Kindl and Schiefer 1971) whereas the aliphatic hydroxyl group is derived by hydroxylation of the corresponding deoxyglucosinolate. Thus 2-hydroxy-3-butenylglucosinolate is derived from 3-butenyl-glucosinolate and the low content of progoitrin in variety Bronowski is due to the presence of a metabolic block at the hydroxylation step (Josefsson 1971).

Overall synthesis process may be summarized as following;

Amino acid → (chain-elongation and homologization) → aldoxime → thiohydroximate → desulfoglucosinolate → glucosinolate (→ hydroxylation; hydroxyglucosinolate).

Catabolism of glucosinolate

The products formed from glucosinolates depend on the

conditions under which they are hydrolyzed. Acid hydrolysis of glucosinolate affords hydroxylamine, sulfate, moieties from the thioglucosidic linkage, and carboxylic acid (R–COOH) (Kjaer 1976). In non-enzymatic hydrolysis the aglucone portion of progoitrin yielded 1-cyano-2-hydroxy--3-butene and 5-vinyl-2-oxazolidinone (Gronowitz et al. 1978) and 3-hydroxypent-4-enethionamide under the influence of ferrous salt (Austin et al. 1968). Sinigrin treated with ferrous salt at 95°C (Youngs and Perlin 1967) or with boiling water (Macleod 1976) yielded allylcyanide and gluconapin yielded 1-cyano-3-butene at 100°C (Gronowitz et al. 1978).

On the other hand, hydrolysis catalyzed by enzyme (myrosinase) takes a different course. In most cases, enzyme-induced detachment of glucose producing the aglucone or its tautomer is followed by molecular rearrangement yielding, with concomitant loss of sulfate, isothiocyanate (R–NCS), nitrile (R–CN) and thiocyanate (R–SCN) depending on the hydrolysis conditions and nature of glucosinolate (Kjaer 1976).

Myrosinase:

Myrosinases are a group of enzymes which are separately deposited in particular cells (idioblast) (Guignard 1890) but invariably accompanying the glucosinolates in the living plant sources. The enzyme was discovered by Bussy as early as 1839. The systematic name is thioglucoside glucohydrolase (E.C. 3.2.3.1.). The question as to whether the hydrolysis

of glucosinolates is carried out with a single enzyme or with a mixture of two entities, a sulphatase and thioglucosidase, has been discussed (Gaines and Goering 1962). Later studies have shown that although myrosinases exist in several multiple forms or isoenzymes they function only as a β-thioglucosidase. Physico-chemical and enzymatic characteristics of pure myrosinase preparations from different plant sources show a small but significant variation among the myrosinase isoenzymes (Tsuruo et al. 1967; MacGibbon and Allison 1970; Björkman and Janson 1972; Henderson and McEwen 1972; Ohtsuru and Hata 1972; Lönnerdal and Janson 1973; Björkman and Lönnerdal 1973). Molecular weights varied from 125,000 to 151,000 for myrosinases and 30,000 to 65,000 for their subunits the number of which varied from 2 to 4 in different myrosinases. Myrosinases were found to be glycoproteins containing from 9 to 23% of carbohydrate (principally hexoses) and had isoelectric points varying from 4.6 to 6.2. The pH optimum for the enzyme activity were between 4 and 7 and reaction rates increased with increasing temperature to 60 - 65°C (Björkman 1976). A study with partially-purified myrosinase preparations showed that myrosinase prepared from *Sinapis alba* has 10 - 30 times as much enzyme activity as those from several rapeseeds (Henderson and McEwen 1972). This result is in accord with the data of Lönnerdal and Janson (1973) who reported that rapeseed (*B. napus*) myrosinase required 15 times more purification (188 vs. 13.6) than white mustard

myrosinase in order to have similar specific activity.

Aglucone Products:

Three pathways for the enzymatic hydrolysis of glucosinolates were suggested by Kjaer (1960);

1. a course involving intramolecular rearrangement to isothiocyanates,
2. an alternative route, predominant in certain plants, that leads to thiocyanates, obviously by rearrangement, and
3. the formation of nitriles and elementary sulphur with no change in the carbon skeleton.

Will and Körner (1863) demonstrated that free sulphur and allyl cyanide, in addition to allylisothiocyanate, are simultaneouly formed upon enzymatic hydrolysis of sinigrin. Ettlinger and Lundeen (1956) proposed that the enzymatic formation of isothiocyanate takes place through Lossen rearrangement. Miller (1965) has demonstrated that the aglucone of sinigrin, generated chemically or enzymatically, decomposes by two competing non-enzymatic pathways; a proton independent isothiocyanate forming one; and a proton dependent nitrile forming one.

Aglucone products of glucosinolates are related to conditions of hydrolysis that are used. In neutral or alkaline media nitriles are formed by enzymatic hydrolysis as compared to non-enzymatic formation of nitrile when the pH of the medium is under 5 (Virtanen 1965). Nitrile formation from progoitrin was promoted by ferrous ion (Fe^{2+})

and special protein fraction (epithio specifier) was responsible for the production of epithionitriles in untreated seed meal (Tookey and Wolff 1970; Tookey 1973a,b). The enzymatic system converting the aglucone of epi-progoitrin to nitriles was labile (Tookey and wolff 1970). The major aglucone product of gluconapin of Yellow Sarson was 1-cyano-3,4-epithiobutane but when the seed was heat treated (115°C for 30 minutes in an oven) this compound almost entirely disappeared and was replaced by a corresponding amount of 3-butenylisothiocyanate on enzymatic hydrolysis (Kirk and McDonald 1974). Macleod (1976) also reported simultaneous formation of isothiocyanate and nitrile from glucosinolates of several *Cruciferae* foodstuffs. He showed that sinigrin is capable of degrading very readily, non-enzymatically as well enzymatically, to give allylcyanide. In order to destroy the factor(s) in the seed meal responsible for the low yield of isothiocyanate, Appelqvist and Josefsson (1967) had to apply more heat (100° - 110°C for 15 min) than that sufficient to inactivate myrosinase (90°C for 15 min).

Gmelin and Virtanen (1959) observed that the enzyme occuring in the seeds and fresh plants of *Thlaspi arvense* L. and *Lepidium ruderale* L. cleaves sinigrin and glucotropaeolin present in those species to allyl- and benzylthiocyanate, respectively. Apparently, no concomitant production of isothiocyanates takes place in these plants, whereas ordinary garden cress (*Lepidium sativum* L.) gives

rise to a mixture of benzylthiocyanate and benzyl-isothiocyanate on autolysis. A third example was provided by Schlüter and Gmelin (1972) who found that extracts of fresh *Eruca sativa* L. plants produced 4-methylthiobutylthiocyanate from 4-methylthiobutylglucosinolate, while the seeds gave the corresponding isothiocyanate. Gmelin and Virtanen (1959) first suggested that the thiocyanate forming reaction involved an enzyme mediated rearrangement. Virtanen and Saarivirta (1962) later proposed that an isomerase acted on a first-formed isothiocyanate, but the second hypothesis has not been supported, while the first remains as a generalized concept. Since the aglucone normally rearranges spontaneously to isothiocyanate, it seems that the rearranging enzyme responsible for the formation of thiocyanate is very efficient, or that the spontaneous isothiocyanate-forming reaction is somehow inhibited, or both (Benn 1977). It was suggested that in *Lepidium* seeds benzylthiocyanate and nitrile were the main products with isothiocyanate only produced when formation of the main products is inhibited (Saarivirta 1973). Tookey (1973b) suggested that a specifier protein similar to epithiospecifier which is needed for the formation of epithionitriles could be reponsible for the formation of thiocyanate.

Gmelin and Virtanen (1960) reported the occurrence of free thiocyanate ion in leaf and root of *Brassica* species. They suggested that isothiocyanate from a certain

glucosinolate may degrade into alcohol and thiocyanate ion. Sinalbin (*p*-hydroxybenzylglucosinolate), and indolyl-glucosinolates glucobrassicin (3-indolylmethylglucosinolate) and neoglucobrassicin (*N*-methoxy-3-indolylmethyl-glucosinolate) readily yielded free thiocyanate ion (Gmelin and Virtanen 1960, 1961, 1962). The presence of substantial amount of thiocyanate ion in rapeseed has been reported by Srivastava and Hill (1975) and McGregor (1978). Sinalbin which was found in some of the rapeseed varieties may be the precursor of the thiocyanate ion in rapeseed (Olsen and Sørensen 1980).

Isothiocyanates have been known to combine with ammonia to form thiourea derivatives. The thiourea derivatives, which have a high ultra-violet (UV) absorbance at 244 nm, were conveniently measured spectrophotometrically (Appelqvist and Josefsson; 1967). Hydroxy isothiocyanates cyclize into vinyl- and allyl-oxazolidinethione (OZT) in a polar solvent. The level of OZT can be readily determined spectrophotometrically based on the absorbance of heterocyclic ring at 248 nm (Wetter 1957). Free thiocyanate ion content, which may be used for indirect estimation of the level of sinalbin and indolylglucosinolates as well, can be determined spectrophotometrically (Johnston and Jones 1966; Josefsson 1968; McGregor 1978). The measurement of total nitrile content was made by infra-red (IR) spectrometry based on the absorption of nitrile functional group near 4.4 μm (Daxenbichler et al. 1967). Determinations

of individual hydrolysis products were made possible by GLC (Youngs and Wetter 1967; Daxenbichler et al. 1970) and the advent of gas chromatography-mass spectrometry (GC-MS) enabled qualitative and quantitative analysis of aglucones to be accomplished simultaneously (Cole 1976; Buttery et al. 1976; Daxenbichler et al. 1979).

B. Anti-nutritional Factors Related to Glucosinolates

Goitrogens cause thyroid hypertrophy by diminishing the supply of thyroid hormone available to the body. This is accomplished either by reducing the supply of iodine available to the thyroid or by interfering with organic binding of the iodide ion which reaches the thyroid. An illustration of the former mechanism is the goiter produced by iodine-deficiency or prolonged thiocyanate administration, while the latter mechanism is exemplified by the action of anti-thyroid drugs such as thiouracil (Morris and Hager 1966).

Aglucone products formed by hydrolysis of glucosinolates are more or less anti-thyroid substances (VanEtten 1969). Enzymatic hydrolysis of progoitrin (2-hydroxy-3-butenylglucosinolate) yields 2-hydroxy--3-butenylisothiocyanate which is unstable and cyclizes to 5-vinyl-2-thiooxazolidone which is also called goitrin due to its strong goitrogenic effect (Kjaer 1960). Thiocyanate ion has been known to act as a goitrogenic compound by lowering iodine concentration while the action of goitrin

may involve the inhibition of the organic binding of iodine in a manner like those of anti-thyroid drugs (Greer 1950; VanEtten 1969). The goitrogenic effect of thiocyanate ion can be prevented or inhibited by increasing the iodine content of the diet (Greer 1950, VanEtten 1969). Isothiocyanate may exert a goitrogenic effect by formation of thiourea derivatives (Greer 1950, 1962) or by formation of thiocyanate ion (VanEtten 1969). Organic nitriles may also act as goitrogens. As with organic isothiocyanate, detoxication of nitriles to form thiocyanate ion offers an explanation (VanEtten 1969). Since drugs of the thionamide series are goitrogenic, thionamide produced by non-enzymatic cleavage of progoitrin (Austin et al. 1968) may act as a goitrogen.

The first definite evidence of the presence of a goitrogen in food was fortuitously discovered in the rabbit fed fresh cabbage (Chesney et al. 1928). Marine et al. (1929) found that goitrogenic factor(s) were also present in other members of the cabbage family or *Cruciferae*. On the assumption that the goitrogenic activity was due to glucosides, Hercus and Purves (1936) were led to test the activity of seeds of various *Brassicae*. They found goitrogenic activity in unsteamed rapeseed and cabbage seed and steamed mustard seeds. Purves (1943) reported that racemic thyroxin in the small dose would abolish the thyroid hyperplasia induced by feeding rats rapeseed. Iodine and diiodotyrosine, on the other hand, would equally modify the

effect of this diet on the basis of their iodide content but would not completely abolish the action, even in large doses. Growth depression and enlargement of thyroid glands was reported when RSMs were fed to chicken (Pettit et al. 1944; Turner 1948) and turkey (Blakely and Anderson 1948). Finally, Astwood et al. (1949) and Carrol (1949) isolated a goitrogenic compound from *Brassica* seeds and identified it as *l*-5-vinyl-2-thiooxazolidone and the structure was confirmed synthetically by Ettlinger (1950). When a high level of goitrin was fed to chickens, I^{131} uptake by the thyroid glands was decreased initially, but returned to normal after 3 to 4 weeks (Clandinin et al. 1966). It was noted that the amount of I^{125} transferred to the egg was reduced when the layers were fed high glucosinolate RSM (Roos and Clandinin 1975, Goh and Clandinin 1977). Addition of thyroprotein to the diet containing RSM did not improve the performance of the chicken although the size of thyroid was drastically reduced (Campbell 1974).

Jackson (1969) noted that layers fed Algerian RSM showed high mortality caused by haemorrhagic liver syndrome (HLS or fatty liver-haemorrhagic syndrome). Death rate was significantly higher in the Hyline 934E strain than in the Hybrid 4. Similar observations have been made by other authors (Clandinin et al. 1974, 1977; March et al. 1975, 1978; Olomu et al. 1975; Slinger 1976a,b; Smith and Campbell 1976; Campbell and Smith 1977). The results obtained generally indicated that HLS is associated with feeding high

levels of RSM particularly those from high glucosinolate rapeseed varieties and that certain strains of chickens, especially the Hyline 934E strain of Leghorns were more suceptible to HLS than others. Hill and Marangos (1974) reported that HLS could not be attributed to OZT since *B. juncea* meal which has no OZT caused a high incidence of HLS. Hall (1972) found that HLS caused by feeding RSM was due to lysis of the reticular substances of the liver affected. Smith and Campbell (1976) supported this finding; however, Yamashiro et al. (1975) reported that hepatocytic degeneration rather than lysis of reticular framework was the major lesion of affected livers. Pearson et al. (1978) reported that incidence of HLS was increased by feeding RSM but without affecting the reticulin content of the liver. Brown (1977) found that the amount of total collagen decreased but the proportion of acetic acid-soluble collagen of total collagen was five times greater in liver of the layers fed Span RSM than in liver of the layers fed a control diet. This suggested that there was a blockage of normal collagen synthesis in livers of birds fed RSM because acetic acid-soluble collagen is usually regarded as a metabolic 'dead-end'. In other disorders of collagen metabolism, such as lathyrism, a disease caused by ingesting nitriles, an abnormal accumulation of acetic acid-soluble collagen similar to that reported was noticed suggesting that a nitrile or nitrile precursor present in the RSM was responsible. Although the specific factor responsible for

HLS has not been definitely identified, accumulated evidence suggests that a hydrolysis product(s) of glucosinolates may be a major factor in the occurrence of HLS (Campbell et al. 1978; Clandinin and Robblee 1978).

Another condition that may be related to glucosinolates is the production of eggs with a fishy odor by some brown-shelled egg layers. Curtis et al. (1978) reported that about 10% of brown shelled-egg layers in commercial flocks lay "tainted" eggs when fed rapeseed meal. It was noted that there was a substantial difference between tainters and non-tainters in their ability to metabolize trimethylamine (TMA) to TMA-oxide which does not cause taint. Thyroid enlargement was significantly greater in tainters than in the non-tainters and thyroid weight was positively correlated with the TMA content of the eggs (Pearson et al. 1978). *In vitro* study with TMA oxidase showed that OZT did not cause significant inhibition of the enzyme. Therefore, it seemed that its effect on TMA oxidation results from an impairment of thyroid function and a consequent reduction in the synthesis of TMA oxidase. Difference in the extent to which individual hens are affected by the goitrogen may also be explained on a genetic basis (Pearson et al. 1979).

The level of goitrin and possibly other aglucone products may influence energy utilization. The ability of chickens to utilize energy of RSM increased with the length of time they were fed a RSM diet before determining the metabolizable energy (ME)of the feed. This suggests that the

adverse effects which hypothyroidism might have on absorbability disappeared as time passed (Lodhi et al. 1969).It was noted that RSM containing a high level of goitrin gave lower ME value for chicks than for laying hens (Lodhi et al. 1969). Addition of goitrin to a SBM-type diet reduced the ME value for young chicks at the level of 0.09% but not at the level of 0.045% of the diet (Lodhi et al. 1970). Muztar et al. (1978) reported that the ME contents of Tower and Candle RSM, which are low in aglucone products, were 2,210 and 2,290 Kcal/Kg DM respectively for Leghorn roosters. March and Soong (1978) reported an ME value for Tower RSM of 2,280 Kcal/Kg DM for 3 week old chicks. These ME values are significantly higher than 1,760 Kcal/Kg (as-fed) for high-glucosinolate RSM suggested previously (Clandinin et al. 1972).

Studies that have been conducted with nitriles suggest that they may have more severe anti-nutritional effects than goitrin. Srivastava et al. (1975) reported that feeding autolyzed RSM which contains a high level of nitrile depressed growth of rats and chickens. Other workers (Tookey et al. 1965; VanEtten et al. 1969a) observed that nitriles produced from autolyzed *Crambe* seed meal were either toxic or fatal when they were administered to rats.

III. EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

Experiments were conducted to study:

Section A; Products of the Hydrolysis of Rapeseed Glucosinolates.

Section B; The Effect of Heat-treatment and Enzyme--hydrolysis of Rapeseed Meal on the Performance of Broiler Chicken.

Secion C; The Effect of Sodium-thiosulfate and Hydroxo--cobalamin on Rats Fed Nitrile-rich or Goitrin-rich Rapeseed Meals.

A. Products of the Hydrolysis of Rapeseed Glucosinolates.

Introduction

Enzymatic hydrolysis of the glucosinolates found in seeds of the *Cruciferae* family yields a variety of substances produced by rearrangement of the aglucon portion. The catabolism of the 2-hydroxy-3-butenyl glucosinolate known as progoitrin or glucorapiferin has long been studied since goitrin (5-vinyl-oxazolidine-2-thione) is formed by spontaneous cyclization of its aglucone portion(a β-hydroxy isothiocyanate) (Kjaer 1960). Epiprogoitrin, which differs only in stereochemical configuration from progoitrin, was isolated from *Crambe abyssinica* (Daxenbichler et al. 1966). Enzymatic hydrolysis of epiprogoitrin ((2S)-hydroxy-3--butenyl glucosinolate), the major glucosinolate of *Crambe* seed, produced (R)-goitrin and (S)-1-cyano-2-hydroxy-3--butene. Two diastereomeric cyano-compounds (2S)-1-cyano-2--hydroxy-3,4-epithiobutanes were also formed from epiprogoitrin (Daxenbichler et al. 1968). Progoitrin from *B. napobrassica* Mill (rutabaga) and *B. napus* yielded the enantiomeric (S)-goitrin and (R)-1-cyano-2-hydroxy-3-butene (Daxenbichler et al. 1966; VanEtten et al. 1966). Isomeric (2R)-1-cyano-2-hydroxy-3,4-epithiobutanes were also formed from progoitrin of *B. napus* meal (Daxenbichler et al. 1967) and *B. campestris* (Lo and Hill 1972).

Besides the above hydroxy glucosinolates the presence of numerous non-hydroxy glucosinolates has been shown. Major aglucone products of non-hydroxy glucosinolates in the raw

seeds of some *Brassica* species were cyano-epithioalkanes (Kirk and MacDonald 1974; Cole 1975, 1976).

The pattern and amount of hydrolysis products of glucosinolates varies with the natures of glucosinolates and myrosinases (Henderson and McEwen 1972; Tookey 1970, 1973a,b) and different hydrolytic conditions (VanEtten et al. 1966; Appelqvist and Josefsson 1967; Tookey 1970). All of the products from the aglucone portion of glucosinolates are, to a greater or lesser extent, goitrogenic or otherwise toxic. Since nitriles are much more toxic than goitrin, which is in turn more toxic than either isothiocyanate (VanEtten et al. 1969a; Srivastava et al. 1975) or thiocyanate, nitriles and goitrin tend to be of greater importance in the utilization of RSMs by livestock.

Information on the hydrolysis products of glucosinolates from rapeseed varieties have been reported by several authors (Lo and Hill 1972; Srivastava and Hill 1974; Smith and Campbell 1976). However, since the products may have far-reaching physiological effects, this experiment was conducted to study the effect of pH, heat treatment, and different enzyme sources on the enzymatic hydrolysis products of rapeseed glucosinolates particularly with respect to the formation of individual nitriles. Non-hydroxy epithionitriles found in the rapeseed varieties are also reported here.

Experimental

A number of glucosinolate-containing meals were

employed in the study including high glucosinolate (Torch and Midas) and low glucosinolate (Candle, Tower and Regent) varieties of rapeseed. Commercial samples of LEAR (low erucic acid rapeseed; derived from a mixture of Torch and Midas seed), *Crambe abyssinica* and rutabaga (*B. napobrassica* Mill) seeds were used to prepare meals with a wide range of glucosinolates.

Preparation of Sample for Gas-liquid Chromatographic (GLC) Determination:

Meals were produced from either raw or heated seed of each variety. The heated seed was prepared by autoclaving the seeds at 110°C for 15 minutes unless specified otherwise. The seeds, either raw or heated, were then ground and extracted with pentane for 48 h to remove the oil. Citrate-Na_2HPO_4 buffers for use in the hydrolysis of the meal were prepared as described by Gomori (1955) for the "1X" buffer and the molarity was doubled for the "2X" buffer. Myrosinase of yellow mustard (*Sinapis alba*) was prepared by the ethanol precipitation method of Appelqvist and Josefsson (1967) and semi-purified rapeseed myrosinases were prepared by the same method but were redissolved and reprecipitated. The samples of meal were then subjected to enzymatic hydrolysis and the products formed were determined by the method of Daxenbichler et al. (1970) with minor modifications. The procedure used was as follows. In the hydrolysis of raw meals (autolysis, i.e., hydrolysis without addition of exogenous myrosinase), 2 ml of 1X buffer

solutions or 2 ml of distilled water was added to 1 g meal. No external source of myrosinase was included. In the hydrolysis of heated meals, 2 ml of distilled water and 10 mg of isolated myrosinase were added to 1 g meal. After thorough mixing, the vial containing the mixture was sealed and incubated at 37°C for 1 h in the case of raw meals and 1 h or longer as specified in the case of heated meals. Following incubation, 30 ml of methylene chloride (CH_2Cl_2) was added and the shaken mixture was filtered through fast filter paper. To 10 ml of the organic phase was added 0.5 mg of internal standard (heptadecanoic acid methyl ester) and the mixture was concentrated under flowing N_2 to approximately 1 ml and the concentrate was used for GLC determination of the hydrolysis products present. Since the pH of the hydrolysates of raw meals with 1X buffer changed, the 2X buffer system was used to maintain constant pH during incubation. To 1 g sample was added 15 ml of 2X buffer (pH 4 or 7) and the mixture was incubated using the same conditions as above. This amount of 2x buffer was the minimum amount required to maintain the desired pH. The hydrolysate was centrifuged (about 5,000 g) and 5 ml of the supernatant was taken to be extracted with 30 ml of methylene chloride along with an internal standard. The aqueous phase of pH 7(2X) hydrolysates tended to form thicker emulsions than those of pH 4(2X) hydrolysates when methylene chloride was used for extraction. The emulsion formed was centrifuged at 20,000 g. The aqueous phase was

reextracted with 15 ml of methylene chloride phase and centrifuged. The methylene chloride phases from both steps were combined and concentrated. The recovery of hydrolysis products was increased by the second extraction in the case of pH 4(2X) buffered hydrolysates but was not increased in the pH 7(2X) buffered hydrolysates. Therefore, a third extraction was conducted on the pH 4(2X) samples.

Gas-liquid Chromatography:

Determinations were made with a Bendix Gas Chromatograph 2500. A glass column of 1.5 m x 0.5 cm was packed with 1% EGSS-X (Applied Science Laboratory Inc.) on Gas-Chrom Q (Applied Science Laboratory Inc.) and conditioned at 200°C with flow of N_2 carrier gas. The column temperature was programmed to hold at 110°C for 3 minutes and then to increase by 5°C per minute to 210°C. The injector, transfer and detector temperature used were 200°C, 230°C and 250°C respectively. Methyl heptadecanoate was used as an internal standard instead of methylpalmitate (Daxenbichler et al. 1970) or methylstearate (Lo and Hill 1972) in order to improve the resolution of peaks of the chromatogram under the conditions used in this experiment. The three internal standards showed the same magnitude of response in the detector. The relative responses of 1-cyano-3,4-epithiobutane and 1-cyano -4,5-epithiopentane were measured to be 58 and 62% of the internal standard respectively. For other compounds the relative responses used by Daxenbichler et al. (1970) were employed.

Isolation and Identification of Nitriles:

For the GLC determination of hydroxy nitriles, standard preparations of (S)-1-cyano-2-hydroxy-3-butene, (2S, 3R)-1--cyano-2-hydroxy-3,4-epithiobutane and (2S,3S)-1-cyano--2-hydroxy-3,4-epithiobutane were obtained from Northern Regional Research Laboratory, U.S.D.A., Peoria, Ill., U.S.A. Goitrin was prepared in our laboratory by the method of Astwood et al. (1949).

In order to identify 1-cyano-3,4-epithiobutane and 1-cyano-4,5-epithiopentane, hydrolysis products of Torch variety of *B. campestris* were used. Unheated Torch seed was crushed and fat was extracted with pentane to prepare the meal. To 100 g of the meal was added 200 ml distilled water and the mixture was incubated for 1 h at 37°C. The hydrolyzed meal was then extracted with 1,000 ml methylene chloride, and filtered. The filtrate was concentrated in vacuum to 5 ml and the precipitate was removed by centrifugation. The supernatant was applied to a silica gel column and eluted with ether-petroleum ether (3:1) as described by Daxenbichler et al. (1966). The concentration of 1-cyano-3,4-epithiobutane plus 1-cyano-4,5-epithiopentane in successive 10 ml fractions of eluate was estimated by silica gel thin-layer chromatography (TLC) developed by iodine vapor. The highest concentrations were detected at about the tenth fraction. Fractions of high concentration were concentrated and subjected to GLC (5% EGSS-X on Gas-Chrom Q) and the peaks for the two nitriles were

collected. Infra-red (IR) spectra of the pure compounds were measured with a Nicolet 7199 FT-IR spectrometer and proton spectra with a FT-NMR. Mass spectra were obtained by high resolution mass spectrometry (M/S 50, AEI) for isolated compounds and by low resolution GC-MS spectrometry (M/S 12, AEI) for complex compounds both using electron impact at 70eV.

Spectrophotometric Determination of Isothiocyanates and Goitrin:

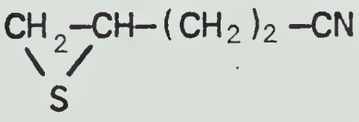
The isothiocyanate and goitrin content of RSMs were determined by the method of Appelqvist and Josefsson (1967).

Results and Discussion

A typical gas chromatograph of the autolysis products of glucosinolates from *B. campestris* 'Torch' variety is shown in Figure III.1. From the chromatograph three hydroxy nitriles (N2, N4 and N5) and goitrin were estimated, but on first separation N1 and N3 were unknown compounds. Torch produced the highest levels of N1 and N3 compounds but similar peaks of lesser magnitudes were found in meals of other rapeseed varieties.

Identification of 1-cyano-3,4-epitiobutane and 1-cyano-4,5--epithiopentane from Rapeseed Meal:

Based on the data obtained from GLC, IR, NMR and mass spectrometry, the unknown compound represented by the peak N1 was identified as 1-cyano-3,4-epithiobutane;



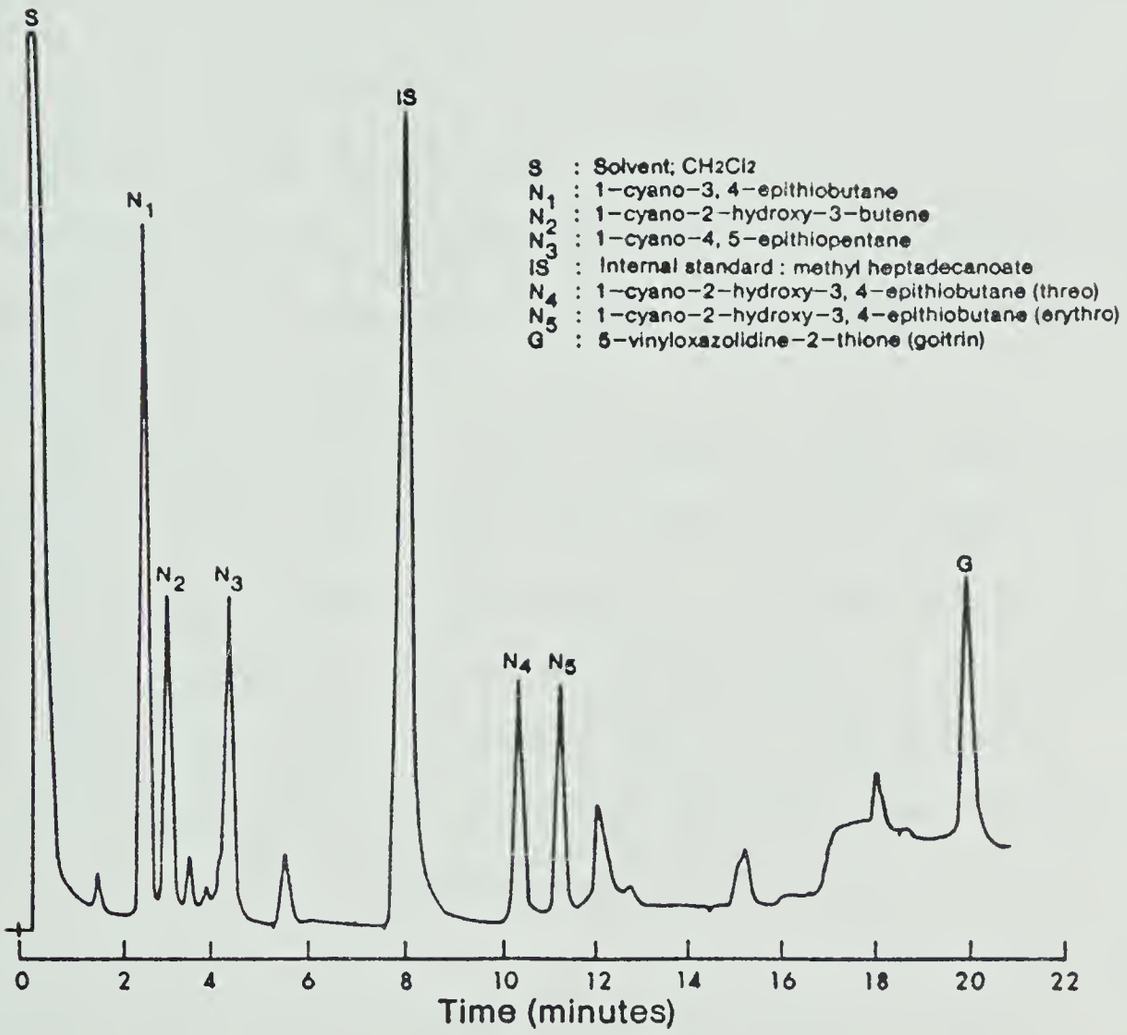
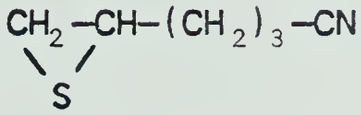


Figure III.1 Chromatograph of hydrolysis products of glucosinolates from raw Torch variety of *B. campestris*

and the peak N3 was identified as 1-cyano-4,5-
-epithiopentane;



The infra-red spectra of 1-cyano-3,4-epithiobutane and 1-cyano-4,5-epithiopentane are shown in Figure III.2 and Figure III.3 respectively. The typical band for the nitrile group occurs at 2246 cm^{-1}. A strong band at 1050 cm^{-1} may be attributed to the wag of the ring methylene in the structure of the compounds (Kirk and Macdonald; 1974). Strong bands around 2950 cm^{-1} and 1425 cm^{-1} may be caused by stretching and scissors vibration of $-CH_2$.

The NMR spectra of the 1-cyano-3,4-epithiobutane and 1-cyano-4,5-epithiopentane are presented in Figure III.4 and Figure III.5. No resonance below δ1.3 indicates the absence of *C*-methyl protons and no resonance above δ3.3 indicates the absence of olefinic double bond. A single proton multiplet centered near δ3.0 can be attributed to the ring proton on C-3 of 1-cyano-3,4-epithiobutane (Kirk and MacDonald 1974) and on C-4 of 1-cyano-4,5-epithiopentane. The peak occuring between δ7 and δ8 results from the internal standard $CDCl_3$. No effort was made to identify the complex peaks between δ1.3 and δ2.75.

Mass spectra of the compounds were measured with both high resolution MS and low resolution GC/MS (Table III.1 and Table III.2). Measured masses were 113.0292 for 1-cyano-3,4-epithiobutane (theoretical mass: 113.0299) and

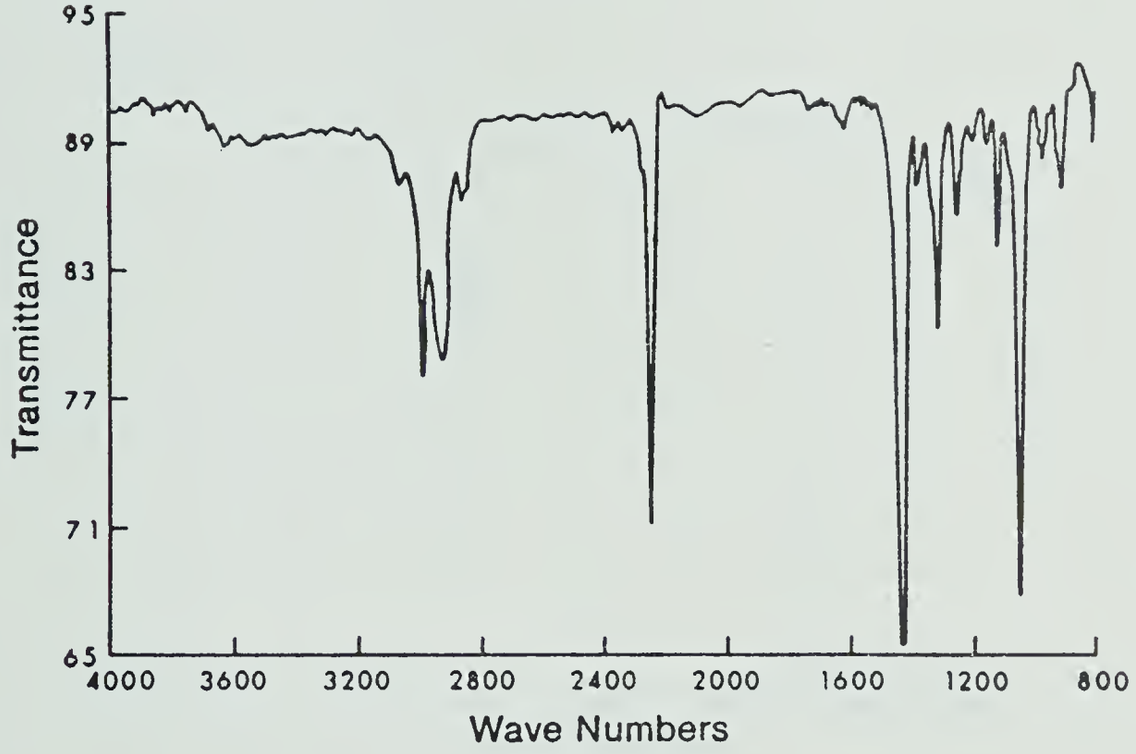


Figure III.2 Infra-red spectrum of 1-cyano-3,4-epithiobutane

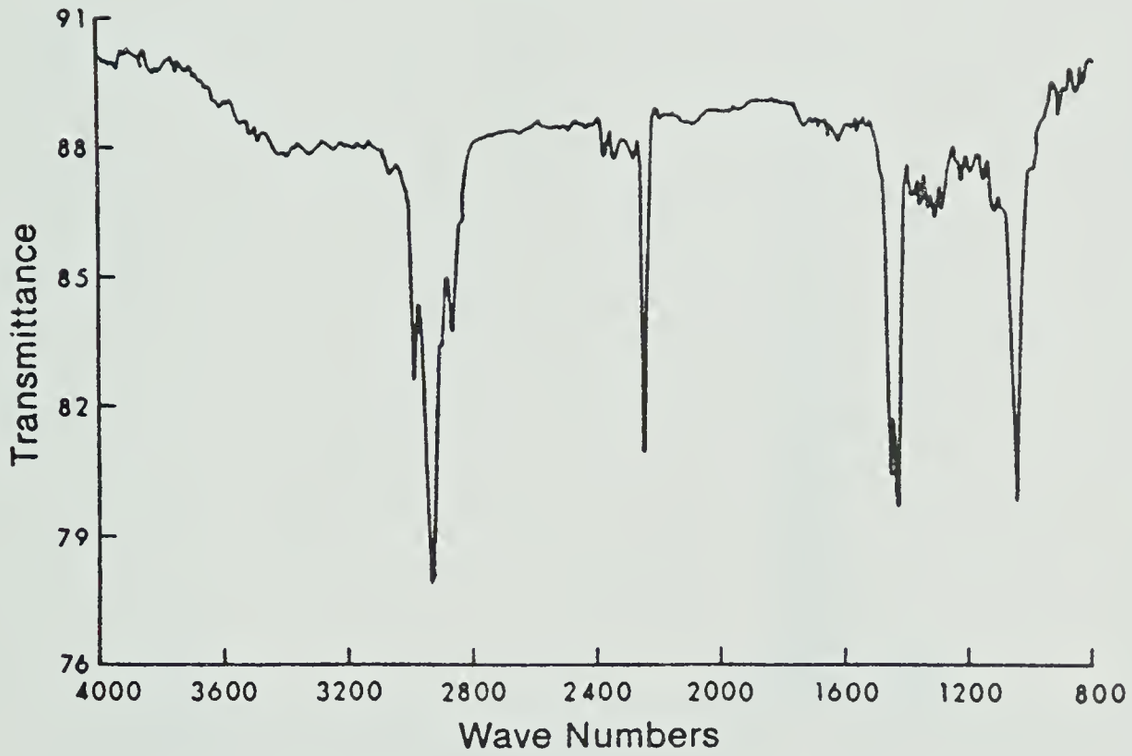


Figure III.3 Infra-red spectrum of 1-cyano-4,5--epithiopentane

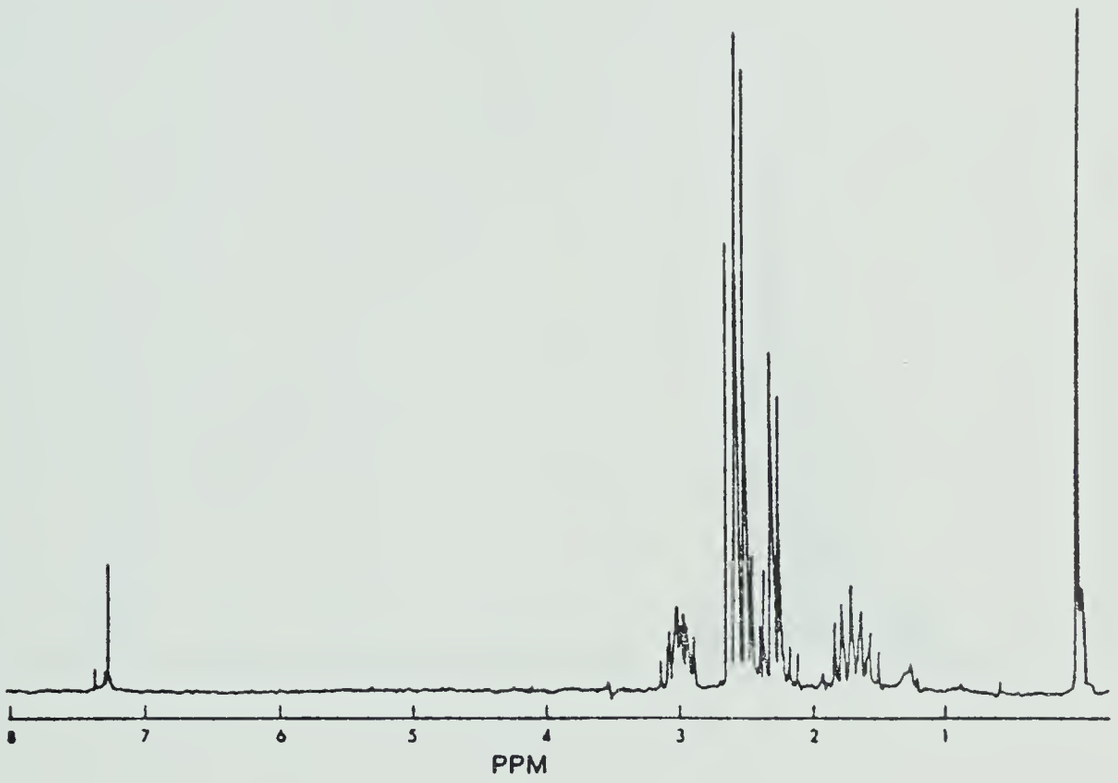


Figure III.4 NMR spectrum of 1-cyano-3,4-epithiobutane

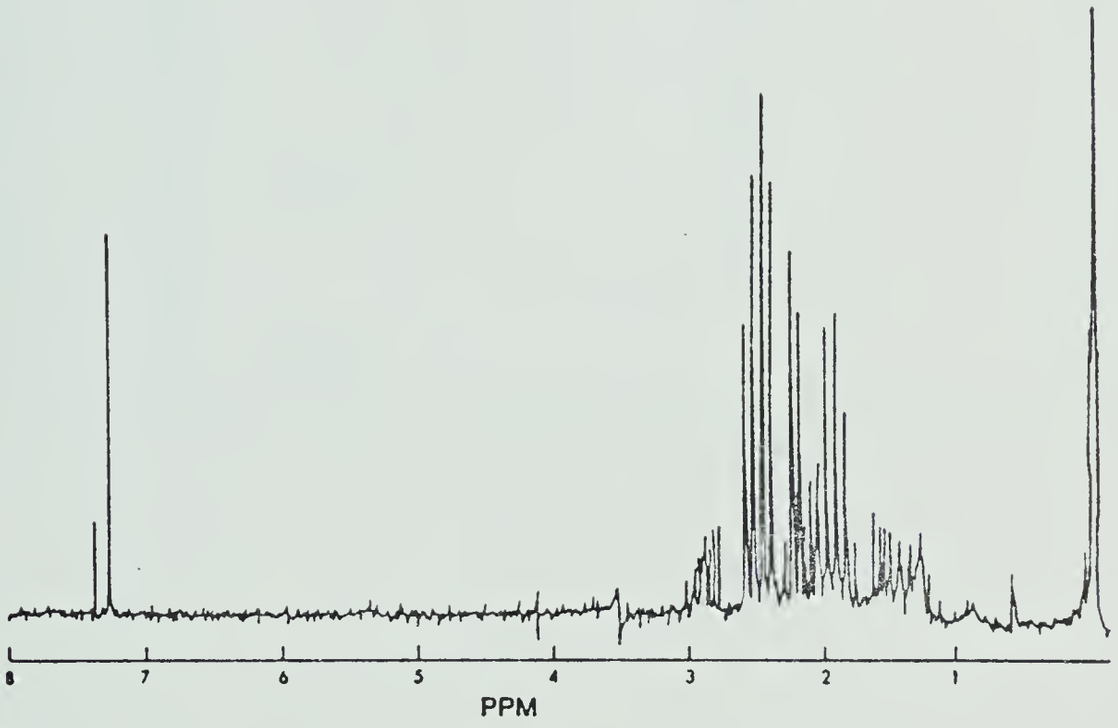


Figure III.5 NMR spectrum of 1-cyano-4,5-epithiopentane

Table III.1 Mass spectrum of 1-cyano-3,4-epithiobutane

M/e	Composition	Relative Intensity	
		High Resol. MS	Low Resol. MS
113	C_5H_7NS	100	25
112	C_5H_6NS	7	1
98	C_4H_4NS	5	1
86	C_4H_6S	35	6
85	C_4H_5S	15	4
81	C_5H_7N	6	20
80	C_5H_6N	22	7
79	C_5H_5N	7	2
73	C_5H_5S	55	15
71	C_3H_3S	0	3
67	C_4H_5N	33	8
60	C_2H_4S	45	14
59	C_2H_3S	0	7
58	C_2H_2S	0	5
54	C_4H_6	12	-
54	C_3H_4N	16	-
54	$C_4H_6 + C_3H_4N$	-	30
53	C_4H_5	29	16
47	CH_3S	-	12
46	CH_2S	-	9
45	CHS	-	28
41	$C_3H_5 + C_2H_3N$	-	100
39	C_3H_3	-	43

Table III.2 Mass spectrum of 1-cyano-4,5-epithiopentane

M/e	Composition	Relative Intensity	
		High Resol. MS	Low Resol. MS
127	C_6H_9NS	100	4
126	C_6H_8NS	13	1
94	C_6H_8N	25	4
87	C_4H_7S	15	2
86	C_4H_6S	13	1
85	C_4H_5S	19	2
82	C_5H_8N	12	2
81	C_5H_7N	12	1
74	C_3H_6S	26	3
73	C_3H_5S	27	3
67	C_5H_7	46	12
66	C_5H_6	20	5
61	C_2H_5S	4	22
60	C_2H_4S	27	4
55	C_4H_7	38	100
54	C_4H_6	19	-
54	C_3H_4S	14	-
54	$C_4H_6 + C_3H_4S$	-	13
53	C_4H_5	17	12
47	CH_3S	-	6
45	CHS	-	10
41	$C_3H_5 + C_2H_3N$	-	88
39	C_3H_3	-	39

127.0455 for 1-cyano-4,5-epithiopentane (theoretical mass: 127.0456). Parent molecular peaks of both compounds were base peaks with high resolution mass spectrometry. The mass spectrum of 1-cyano-3,4-epithiobutane closely matched that obtained by Kirk and MacDonald (1974) who isolated this compound from the autolysis products of glucosinolate (3-butenyl glucosinolate) of Yellow Sarson (*B. campestris*). The spectrum of 1-cyano-4,5-epithiopentane was also in good agreement with the spectra published by Cole (1975) who isolated this compound from a number of members of the *Cruciferae* family. Based on the data obtained here and their similarity to published data, it was concluded that the compounds isolated from Torch RSM were 1-cyano-3,4-epithiobutane and 1-cyano-4,5-epithiopentane.

It is likely that 1-cyano-3,4-epithiobutane and 1-cyano-4,5-epithiopentane are derived from gluconapin (3-butenyl glucosinolate) and glucobrassicanapin (4-pentenyl glucosinolate) respectively because the presence of these glucosinolates in the seed of *B. napus* and *B. campestris* is known (Josefsson 1970). Other nitriles that might have been formed are 1-cyano-3-butene from gluconapin and 1-cyano-4-pentene from glucobrassicanapin because their presence in autolyzed RSM (Anjou 1978) and crude rapeseed oil (George and Töregård 1978) has been reported but their presence was not detected in the present GLC system perhaps because of their low boiling point.

Influence of Hydrolysis Conditions on the Formation of Nitriles and Goitrin:

Effect of pH.

In an initial study, the pHs of hydrolysates of raw and heated meals were measured. The pHs of a number of raw rapeseed, *Crambe* and rutabaga seed meal preparations after autolysis in distilled water ranged from 5.00 to 5.72. The pHs of heated RSM preparations after incubation with mustard myrosinase ranged from 5.14 to 5.65 (Table III.3). The hydrolysates of high glucosinolate varieties had lower pHs (Crambe 5.00; rutabaga 5.10; Midas 5.15 for raw meal and 5.14 for heated meal) than those of low glucosinolate varieties (Candle 5.72 and 5.65; and Tower 5.75 and 5.65, for raw and heated meal respectively). Heated meals hydrolyzed with mustard myrosinase tended to have a slightly lower pH than those of raw meals.

A possible explanation of the pH differences in hydrolysates of high and low glucosinolate varieties is that high glucosinolate varieties (or species) release more acid sulfate (Croft 1979). Hydrolysis of heated meals with mustard myrosinase must have released slightly more acid sulfate from glucosinolates than autolysis of raw meals because the pH of the former was slightly but consistently lower. As the pH of hydrolysates in the present study was approximately 5.4, two extremes, pH 4 and 7, were chosen for study. The pH of autolysis samples with 1X buffer solution increased during 1 h of incubation from the initial 4 to

Table III.3 pH of hydrolysates of seed meals

Variety & Species	Raw Meal[1]	Heated Meal[2]
Candle (*B. campestris*)	5.72	5.65
Torch (*B. campestris*)	5.46	5.21
Midas (*B. napus*)	5.15	5.14
Regent (*B. napus*)	5.50	5.41
Tower (*B. napus*)	5.75	5.65
LEAR[3]	5.30	-
Crambe abyssinica	5.00	-
Rutabaga	5.10	-

[1] Autolyzed for 1 h at 37°C with 3 ml of distilled water.
[2] Hydrolyzed with mustard myrosinase under the same conditions as raw meal.
[3] Low erucic acid rapeseed; derived from a mixture of Torch and Midas seed.

approximately 5 for samples with an initial pH of 4 and decreased from 7 to approximately 6 for samples with an initial pH of 7. Thus, in the interpretation of data, autolysis products with pH 4(1X) buffer may have been obtained at pH 5 and those with pH 7(1X) may have been obtained at pH 6.

The effect of differing pH and buffer concentrations on the autolysis products from raw meals of rapeseed, *Crambe abyssinica* and rutabaga are presented in Table III.4. In most instances, autolysis of raw meal produced mainly nitriles, and a small amount of goitrin. This was similar to the observation of Daxenbichler et al. (1968, 1970) with fresh and wetted *Crambe* meal. In the case of rutabaga and Midas meal C, however, the levels of goitrin produced were greater than the levels of total nitriles. Acidic condition (pH 4, either 1X or 2X) favored the production of total nitrile in autolyzed Candle, Torch and Tower meals. Levels of 1-cyano-3,4-epithiobutane and 1-cyano-4,5-epithiopentane were most sensitive to the change of pH. Total nitrile production of the Regent hydrolysate did not show noticeable difference when pH 7 or pH 4 was used in the autolysis, but the production of 1-cyano-3,4-epithiobutane and 1-cyano-4,5--epithiopentane was greater under acidic conditions. The highest level of total nitrile production occurred with Midas meal while Torch showed the highest level of non-hydroxy epithionitrile. In general, the non-hydroxy epithionitriles showed more sensitivity to the change of pH

Table III.4 Autolysis products of glucosinolates of raw seed meals at different pH and concentration of buffer (mg/g meal)[1]

Variety or Species	Buffer	Nitriles[2]					Total Nitrile	Goitrin
		N1	N2	N3	N4	N5		
Candle	pH 4(1X)[3]	0.46	0.20	0.25	0.25	0.25	1.41	0.26
	pH 7(1X)[3]	0.01	0.21	0	0.24	0.24	0.70	0.15
	pH 4(2X)	0.34	0.16	0.19	0.16	0.16	1.01	0.20
	pH 7(2X)	0.06	0.18	0	0.18	0.17	0.59	0.18
Torch	pH 4(1X)	2.22	0.50	1.93	0.59	0.60	5.84	1.10
	pH 7(1X)	1.60	0.44	0.98	0.79	0.73	4.54	1.04
	pH 4(2X)	1.10	0.41	1.07	0.48	0.47	3.53	0.77
	pH 7(2X)	0.62	0.31	0.25	0.44	0.43	1.12	0.67
Regent	pH 4(1X)	0.38	0.42	0.03	0.58	0.57	1.98	0.38
	pH 7(1X)	0.16	0.53	0	0.58	0.59	1.86	0.35
	pH 4(2X)	0.26	0.34	0.02	0.44	0.49	1.53	0.28
	pH 7(2X)	0.06	0.40	0	0.55	0.53	1.54	0.28
Midas A	pH 4(1X)	2.05	2.18	0.23	2.28	2.17	8.91	0.49
	pH 7(1X)	2.19	2.40	0.26	2.43	2.34	9.62	0.49
	pH 4(2X)	0.99	1.74	0.10	1.51	1.62	5.96	1.11
	pH 7(2X)	1.13	1.58	0.13	1.09	1.09	5.02	1.76
Midas B	Water	1.92	2.30	0.16	1.91	1.81	8.10	1.45
Midas C	Water	0.71	1.94	0.10	0.96	0.92	4.63	10.23
	pH 5.4(1X)	0.85	1.53	0.12	1.11	1.12	4.73	9.66
	pH 5.4(2X)	0.74	1.42	0.10	0.86	0.88	4.00	9.99
Tower A	pH 4(1X)	0.95	0.33	0.56	0.44	0.40	2.68	0.58
	pH 7(1X)	0.65	0.31	0.26	0.43	0.39	2.04	0.56
	pH 4(2X)	0.50	0.22	0.29	0.26	0.27	1.54	0.45
	pH 7(2X)	0.40	0.19	0.17	0.30	0.28	1.34	0.58

continued-

Variety or Species	Buffer	Nitriles[1]					Total Nitrile	Goitrin
		N1	N2	N3	N4	N5		
continued-								
Tower B	Water	0.39	0.33	0.03	0.30	0.29	1.34	0.33
Tower C	Water	0.08	0.21	0	0.13	0.13	0.55	0.37
	pH 5.4(1X)	0.11	0.13	0	0.16	0.16	0.56	0.38
	pH 5.4(2X)	0.09	0.21	0	0.15	0.14	0.59	0.43
LEAR	Water	0.78	1.90	0.39	1.15	1.33	5.55	0.86
Crambe	Water	0.06	3.16	0	3.89	4.84	11.95	0.48
Rutabaga	Water	0.22	2.11	0	0.34	0.44	3.11	10.32

[1] Average value of duplicate samples.
[2] Initial pH 4(1X) and pH 7(1X) changed to pH of approximately 5 and 6 respectively after incubation for 1 h.
[3] N1: 1-cyano-3,4-epithiobutane
N2: 1-cyano-2-hydroxy-3-butene
N3: 1-cyano-4,5-epithiopentane
N4: 1-cyano-2-hydroxy-3,4-epithiobutane (threo)
N5: 1-cyano-2-hydroxy-3,4-epithiobutane (erythro)

than the hydroxy nitriles. The pattern of hydrolysis products of Midas meal A (Figure III.6) is in agreement with the pattern of Meal A of VanEtten et al. (1966) and Meal B of Tookey and Wolff (1970) who referred to it as the "fresh meal pattern".

While the patterns of goitrin production in other rapeseed meals (e.g. Torch in Figure III.6) were not markedly influenced by pH, those of the total nitrile production are similar to that of Midas meal A which produced the highest levels of total nitrile and the lowest levels of goitrin at pH 5 to 6. The optimum pH for nitrile production from endogenous enzymatic hydrolysis of glucosinolates present in the complex system differed from that reported by Daxenbichler et al. (1966) for nitrile production in an isolated system in which purified epiprogoitrin was hydrolyzed by mustard myrosinase. In the isolated system the optimum pH for the production of nitrile was approximately 3 and minimum production occurred at pH 5-9, while the reverse held for the production of goitrin. Differences in amounts and pattern of hydrolysis products within different samples of the same variety (e.g. Midas or Tower) may be attributed partly to the different aging period and to variations within the variety. Increasing the concentration of buffer solution at pH 5.4 (the average pH of hydrolysates with water) did not show any consistent influence on the pattern or the amount of autolysis products. However, it was observed that at the pH 4 and 7

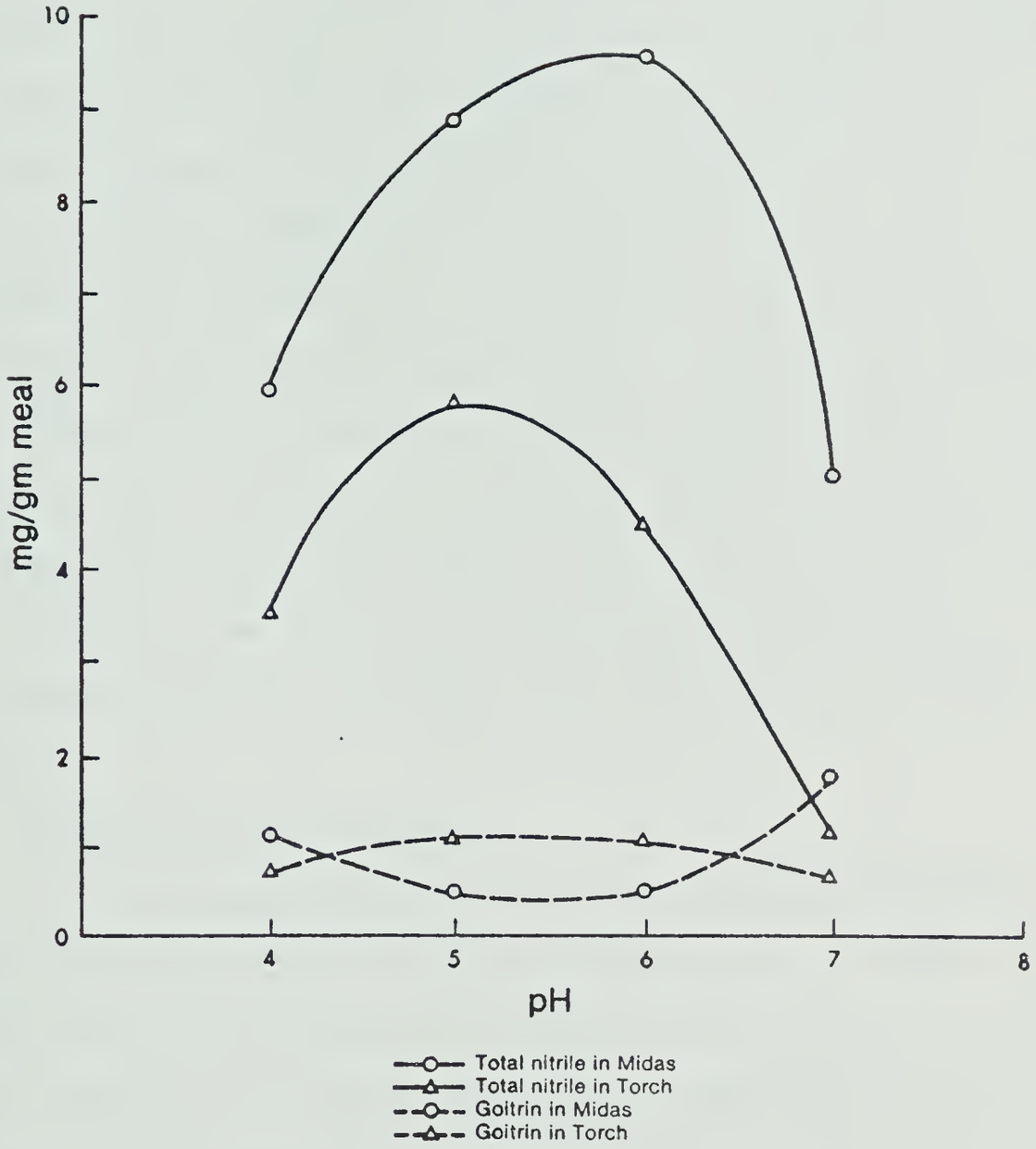


Figure III.6 Autolysis products of raw rapeseed glucosinolates at different pHs (Midas and Torch)

with 2x buffer, less total nitriles and goitrin were produced than at the intermediate pH with 1x buffers that were not strong enough to maintain the initial pH. As the myrosinase activity of Turret variety of *B. napus* (Henderson and McEwen 1972) was maximum around pH 5.5, and was reduced greatly at pH 4 and 7 with a citrate-Na_2HPO_4 buffer system, it is more likely that the reduced total production of nitriles and goitrin is caused by unfavorable pH for enzyme activity rather than increased concentration of buffers. Although the nitriles and goitrin measured here do not account for all of the products from aglucone rearrangement, they showed differences in the total amount of aglucone products under present experimental conditions. However, it was reported that total glucosinolates hydrolyzed was not influenced by the change of pH during hydrolysis when it was measured by titration of HSO_4 ion liberated (VanEtten et al. 1969b).

Effect of heat treatment.

As expected from the observations of Daxenbichler (1966) with epiprogoitrin and VanEtten et al. (1966) with *Crambe* seed, the production of goitrin was lowest in raw meals (Table III.4 and Figure III.6). When RSMs prepared from heated rapeseed were autolyzed only a trace of aglucone products was detected regardless of varying heat-treatments (Table III.5). In order to destroy the factor(s) responsible for low yield of isothiocyanate Appleqvist and Josefsson (1967) had to heat rapeseed at 100° - 110°C for 15 miniutes

Table III.5 Autolysis products of glucosinolates of rapeseed meals prepared from seeds treated under different conditions of time and temperature (mg/g meal)[1]

	Heat-treatment				
Rapeseed	Temp.	Time	Container	N2[2]	Goitrin
Torch	110°C	5 min.	Autoclave	0.03	0.11
	110°C	15 min.	Autoclave	0.05	0.48
	110°C	15 min.	Closed vial	0.02	0.06
	110°C	30 min.	Autoclave	0.07	0.14
	121°C	30 min.	Autoclave	0.18	0.40
Midas	110°C	5 min.	Autoclave	0.06	0.14
	110°C	15 min.	Autoclave	0.13	0.06
	110°C	15 min.	Closed vial	0.10	0.01
	110°C	30 min.	Autoclave	0.06	0.39
	121°C	30 min.	Autoclave	0.10	0.18

[1] Average value of duplicate samples.
[2] 1-cyano-2-hydroxy-3-butene. Other nitriles are detected only in a trace amount (<0.01).

in a closed vessel. All heat-treatments tested in the present study were sufficient to inactivate myrosinase and produce minimum amounts of aglucone products on autolysis. Small amounts of 1-cyano-2-hydroxy-3-butene and goitrin were consistently detected in those meals. Since the increased temperature and time of treatment of seed did not further reduce the amount of aglucones present in RSM, a low level of hydrolysis products may have been present in the rapeseed prior to processing. Based on these results, in further studies heated RSMs were prepared by autoclaving seeds at 110°C for 15 min.

On hydrolysis of heated RSMs with exogenous myrosinase, the products of the glucosinolates show a completely different pattern than those produced by glucosinolates in raw seed meals. In the present assay of hydrolysis products of glucosinolates from heated rapeseed meals, no effort was made to maintain any specific pH but the pH of the hydrolysates averaged 5.4 (Table III.3). Table III.6 and Table III.7 show the concentration of hydrolysis products of meals prepared from heated seeds of different varieties. The level of goitrin was much higher than that of isothiocyanate in either Tower or LEAR meal (Table III.6). Since the level of isothiocyanate determined here is the sum of all the volatile isothiocyanates, progoitrin is more abundant than the volatile isothiocyanate-producing glucosinolates in those RSMs. Incubation of heated seed meals with myrosinase produced only small amounts of nitriles regardless of

Table III.6 Level of isothiocyanate and goitrin in heated Tower and LEAR meal hydrolyzed with mustard myrosinase (mg/g meal)[1]

Variety	Isothiocyanate	Goitrin
Tower	0.67	1.42
LEAR[2]	2.68	7.82

[1] Average value of duplicate samples analyzed by the method of Appelqvist and Josefsson (1967).
[2] Low erucic acid rapeseed; derived from a mixture of Torch and Midas seed.

Table III.7 Hydrolysis products of glucosinolates from heated rapeseed meal with different sources of myrosinase and incubation time (mg/g meal)[1]

Variety	Source of Enzyme	Incubat'n Time (h)	Nitriles[2]					Total Nitrile	Goitrin
			N1	N2	N3	N4	N5		
Candle	Mustard myros.	1	0	0.01	0.02	0	0	0.03	1.04
	Candle myros.	1	o	0.01	0.01	0	0	0.02	0.40
	Candle myros.	2	0	0.01	0.01	0	0	0.02	0.86
Torch	Mustard myros.	1	0	0.03	0.15	0	0	0.18	3.03
	Torch myros.	1	0	0.02	0	0	0	0.02	0.50
	Torch myros.	2	0	0.02	0.01	0	0	0.03	0.55
Regent	Mustard myros.	1	0	0.08	0	0.02	0.03	0 13	2.50
	Regent myros.	1	0	0	0	0	0	0	0.38
	Regent myros.	2	0	0.03	0	0	0	0.03	0.74
Midas	Mustard myros.	1	0	0.20	0.02	0	0.01	0.23	9.95
	Midas myros.	1	0	0.12	0	0	0	0.12	0.63
	Midas myros.	2	0	0.12	0	0	0	0.12	1.22
	Midas myros.	24	0	0.22	0	0	0	0.22	8.43
Tower A	Mustard myros.	1	0	0.03	0.06	0.01	0	0.10	1.66
	Tower myros.	1	0	0.02	0.01	0	0	0.03	0.38
	Tower myros.	2	0	0.02	0.03	0	0	0.05	0.78
Tower B	Ground raw Tower seed								
	5%	12	0	0.04	0	0	0	0.04	1.58
	10%	12	0.01	0.05	0	0.01	0.01	0.08	1.66
	5%	24	0	0.03	0	0	0	0.03	1.72
	10%	24	0	0.04	0	0	0	0.04	1.58
	5%	36	0	0.04	0	0	0	0.04	1.57
	10%	36	0	0.04	0	0	0	0.04	1.55

[1] Average value of duplicate samples.
[2] Refer to Figure III.1 or Table III.4 for nitriles (N1-N5).

sources of enzymes (Table III.7). It appears that almost all of the aglucone portion of progoitrin was converted to goitrin and that the aglucone portion of non-hydroxy glucosinolates such as gluconapin and glucobrassicanapin were converted to isothiocyanate. The major nitrile produced from heated meal was 1-cyano-2-hydroxy-3-butene but it was produced in very small amounts.

Influence of source of enzyme.

The myrosinase obtained from mustard seed was much more active than semi-purified myrosinases prepared from rapeseed. Incubation of heated seed meals for 1 h with mustard myrosinase yielded from 2.5 (Candle) to 15 (Midas) times more goitrin than with semi-purified rapeseed myrosinases (Table III.7). The higher activity of mustard myrosinases noted as compared to semi-purified rapeseed myrosinases agrees with the observations of Henderson and McEwen (1972) and Lönnerdal and Janson (1973).

The yield of goitrin after incubation of heated meal with myrosinase derived from Midas meal increased as a function of incubation time reaching a plateau in 24 - 36 h (Figure III.7). When ground raw Midas or Tower seed was added as a source of enzyme, maximum production of goitrin occurred within 12 h incubation with 5 or 10% added seeds (Figure III.7 for Midas and Table III.7 for Tower). When 1% mustard myrosinase was used, maximum production of goitrin had already occurred after 1 h of incubation. Some inactivitation of the isolated rapeseed myrosinase may have

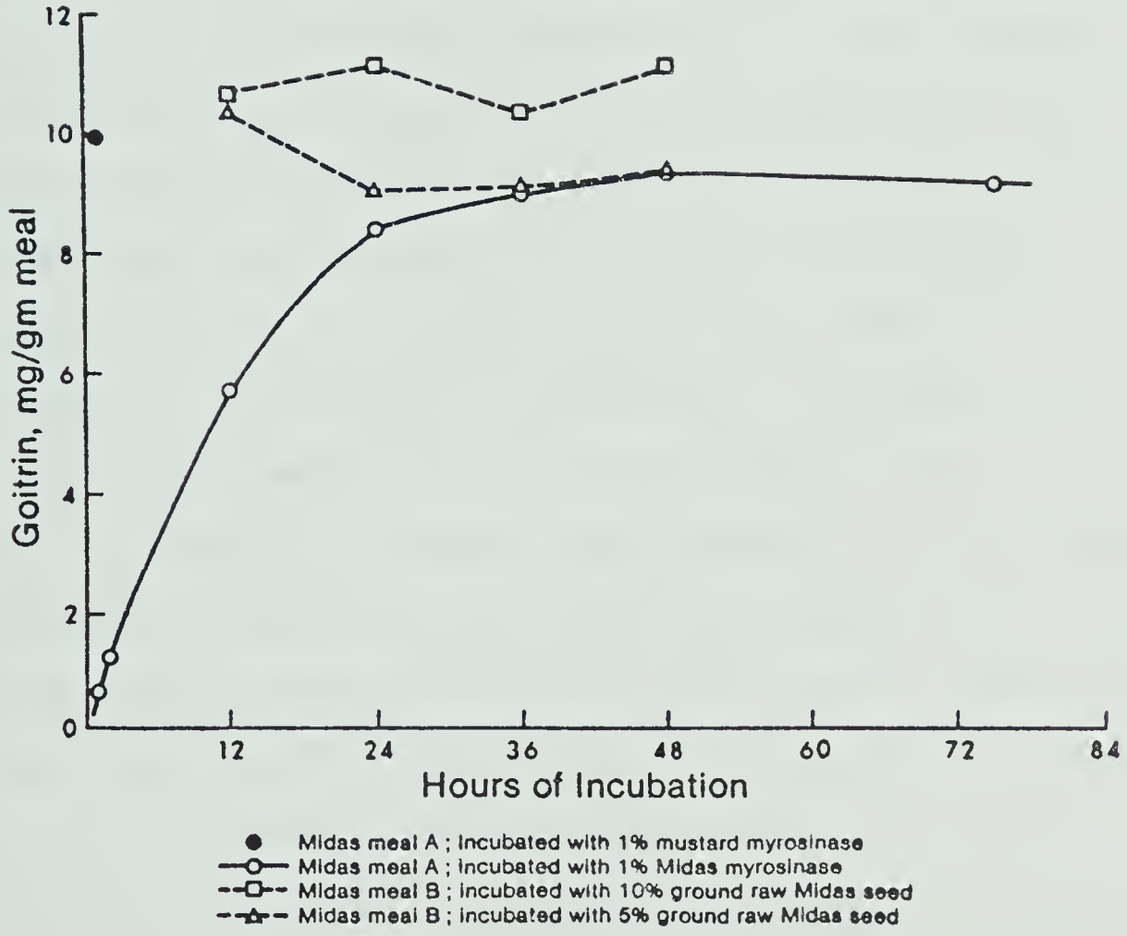


Figure III.7 Goitrin production of heated Midas meal with different myrosinase sources

occurred in the preparation due to oxidation because the crude enzyme requires a reducing agent such as mercaptoethanol or ascorbate for stability (Tookey 1973a).

The hydrolysis pattern of glucosinolates is complex. For the production of 1-cyano-2-hydroxy-3-butene, a particular enzyme fraction and ferrous ion was required (Tookey 1970, 1973a,b). In addition to myrosinase another protein referred to as ESP (epithio specifier protein) was required to promote the formation of 1-cyano-2-hydroxy-3,4--epithiobutanes (Tookey 1973b). A hypothesis by Tookey and Wolff (1970) to account for the complex products formed in *Crambe* was that the factor(s) required for production of cyano-compounds is easily oxidized and unstable. Since ESP is unstable and subject to oxidation, the *Crambe* enzymatic system may be destroyed by extreme change in pH, by aging of meal, by aging in solution, but the most of all by heat treatment. The same factors may have affected the results observed with rapeseed meals in this study. In addition, hydroxy epithionitriles produced from progoitrin easily undergo polymerization (Daxenbichler et al. 1968) by prolonged incubation time or during storage of compounds. Such instability may also have affected the results of this study.

Summary

A study was conducted to identify the products, mainly nitriles and goitrin, of enzymatic hydrolysis of glucosinolates present in meals of a number of rapeseed

varieties.

The pH of autolysates of meals from seeds of the *Cruciferae* family ranged from 5.00 to 5.72. High glucosinolate varieties had lower pHs than low glucosinolate varieties. On autolysis of raw meals, the main hydrolysis products were nitriles with maximum production occurring at about pH 5 to 6. The most stable nitrile when hydrolysis conditions were changed was 1-cyano-2-hydroxy-3-butene. The presence of the nitriles, 1-cyano-3,4-epithiobutane and 1--cyano-4,5-epithiopentane, in rapeseed meal hydrolysates was confirmed by gas-liquid chromatography, infra-red spectrometry, nuclear magnetic resonance and mass spectrometry. Both nitriles were found in Candle, Torch, Regent, Midas and Tower RSMs. Goitrin was the predominant product from heat treated meal regardless of source of myrosinase enzyme. The enzyme activity of semi-purified myrosinase of RSMs was 6 to 40% of that of mustard myrosinase.

B. The Effect of Heat-treatment and Enzyme-hydrolysis of Rapeseed Meal on the Performance of Broiler Chicken.

Introduction

Hydrolysis of glucosinolates of rapeseed or RSMs has a great influence on their nutritive value and the influence appears to be related to the levels and kinds of glucosinolates in different varieties of rapeseed and the hydrolysis conditions used. Among the hydrolysis products formed, nitriles and goitrin exert the greatest toxic effect. Feeding rations containing 28% autolyzed *Crambe* seed meal to rats caused death in all rats within 2 weeks (Tookey et al. 1965). VanEtten et al. (1969a) demonstrated that either autolyzed *Crambe* meal, identified as being rich in hydroxy nitriles, or the nitrile mixture itself was more harmful to rats than goitrin. In the experiment nitriles caused poorer growth or death, and resulted in enlargement of livers, kidneys and thyroid glands and produced histological lesions. Rats fed meal containing intact epiprogoitrin plus active myrosinase had similar, but milder, lesions than rats fed the nitrile mixture. A similar study conducted with rats and chickens using high glucosinolate RSM showed that nitrile-rich RSM was more toxic than goitrin-rich RSM and that rats were more severely affected than chickens (Srivastava et al. 1975). A combined level of potential goitrin and isothiocyanate up to 1 mg/g of diet had no influence on the weight gain of mice (Josefsson and Munck 1973) but the same level of nitriles in

the diet could be harmful because heat treatment improved growth rate of mice.

The beneficial effect of heat treatment on the nutritive value of high glucosinolate rapeseed has long been recognized (Bayley and Summers 1975). Heat-treatment inactivates factor(s) that convert epiprogoitrin to cyano compounds (Tookey and Wolff 1970; Josefsson 1975a) as well as inactivating myrosinase. As a result of heat treatment, progoitrin is converted to goitrin which is less toxic than nitriles upon hydrolysis with exogenous myrosinase.

While Leeson et al. (1978) found that layers or broilers did not respond to heat treatment of low glucosinolate Tower rapeseed others (Lo and Hill 1971; Josefsson and Munck 1972; Josefsson 1975b) noted that mice did respond to this treatment of low glucosinolate Bronowski RSMs.

Since studies on the nutritive value of RSMs have provided limited information on the combined influence of variety, heat treatment and method of hydrolysis, and the content of hydrolysis products of rapeseed glucosinolates, an experiment was undertaken with broiler chicken to study the effect of these factors.

Experimental

Marerials and Methods:

In the experiment seeds of two varieties of rapeseed, either raw or heated, were defatted and hydrolyzed in different ways. The treatments were designed to produce

meals that contained or had the potential to produce varying levels of nitriles or goitrin in the rations fed to broiler chickens. A portion of each of two varieties of *B. napus*, (Midas, a high glucosinolate variety and Tower, a low glucosinolate variety), was heated in the autoclave at 110°C for 15 minutes while the rest was unheated.

The rapeseed samples, either raw or heated, were ground coarsely and the oil was extracted in a Soxhlet apparatus for 24 h using hexane. The solvent was removed from the meals by drying under a fume hood. Meals thus prepared were then divided into three parts and treated in one of three ways. One portion of each meal was thoroughly mixed with 10% ground raw rapeseed as a source of myrosinase and mixed 1:2 (w/w) with distilled water. The mixture was then incubated at 37°C for 24 h to allow complete hydrolysis of the glucosinolates present. After incubation, the hydrolyzed RSM was cooled and freeze-dried under vacuum. These meals were referred to as pre-hydrolyzed freeze-dried (PHFD) rapeseed meal. The PHFD meals were added to the rations at a level of 22%. The second portion of each variety of RSM was mixed in the ration at a level of 20% along with 2% ground raw rapeseed to serve as a source of enzyme for possible hydrolysis of the glucosinolates in the digestive tract. The third portion of each RSM was mixed in the ration at a level of 20% along with 2% ground heated rapeseed and therefore provided no myrosinase for hydrolysis of the glucosinolates in the heated meals ingested. An isocaloric and

isonitrogenous ration containing soybean meal served as a control.

Two hundred and sixty day-old, cross-bred male broiler chickens (White Mountain male x Hubbard female) were divided at random into 26 groups of 10 birds each. Two groups were placed on each of the experimental rations (Table III.8). The individual treatments are detailed in Table III.10. Feed and water were supplied *ad libitum*.

The chicks were group weighed at 0, 7, 14 and 21 days of age and individually at 28 days when the experiment was terminated. A record of feed consumption, mortality and leg abnormalities was kept. Upon completion of the experiment, 4 birds from each group were selected at random, killed by cervical dislocation and their thyroid glands were removed and weighed.

For the statistical analysis of the results, analysis of variance and Duncan's Multiple Range Test (Steel and Torrie 1960) were used to test for significance ($p<0.05$) between treatment means.

Analysis of Nitriles and Goitrin:

Levels of nitriles and goitrin in the RSM samples were determined using GLC method of Daxenbichler et al. (1970) with minor modifications described in previous section. Samples for GLC determination of potential aglucone levels were prepared either by hydrolysis with exogenous enzyme or by autolysis with endogenous enzyme. Hydrolysis of heated RSM was conducted by adding 10% ground raw rapeseed as a

Table III.8 Composition of diets of broiler chicken

Ingredients	Rapeseed	Control
	%	%
Ground wheat	59.2	60.7
Herring fish meal (72%)	5.0	5.0
Meat meal (50%)	5.0	5.0
Rapeseed meals	20.0	-
Ground rapeseed	2.0	-
Soybean meal (47.5%)	-	13.0
Stabilized animal fat	7.0	6.0
Wheat shorts	-	8.5
Limestone	0.6	0.6
Iodized salt	0.2	0.2
Micromix[1]	1.0	1.0
Calculated composition:		
Metabolizable energy (MJ/kg)	12.69	12.68
Crude protein (%)	21.9	21.9
Ca (%)	0.92	0.96
P (%)	0.74	0.81

[1] Supplied the following levels per kg diet: Mn 126 mg; Zn 81 mg; Se 0.1 mg; vitamin A 3000 IU; vitamin D 600 ICU; vitamin E 10 IU; menadion sodium bisulfite 1 mg; Riboflavin 4 mg; Ca-pantothenate 5 mg; niacin 20 mg; biotin 0.2 mg; choline-chloride 77 mg; vitamin B_{12} 0.01 mg; D-L methionine 227 mg; Amprolium 125 mg.

source of myrosinase and mixing 1:2 (w/w) with distilled water. A previous study (Section A) indicated that this level of ground raw rapeseed was adequate to hydrolyze the glucosinolates in RSM. The mixture was then incubated at 37°C for the lengths of time specified. Autolysis of rapeseed, either raw or heated, was conducted under the same condition as outlined above but without adding an exogenous source of myrosinase. Since the hydrolysis products of glucosinolates in PHFD RSM were not directly extracted into the organic solvent phase (methylene chloride), the PHFD meals were mixed with distilled water (1:2, w/w) and kept for one hour at 37°C, after which the mixture was extracted with methylene chloride. In order to characterize a unknown compound which appears during GLC determination of PHFD meals GLC, IR and mass spectrometry were used.

Results and Discussion

Levels of Nitriles and Goitrin in Treated RSMs:

Concentrations of nitriles and goitrin in the samples of Tower and Midas meals are shown in Table III.9. In general, autolysis of raw meal resulted in high levels of nitriles and low levels of goitrin. Because of the higher levels of glucosinolates in Midas meal the level of nitriles was considerably higher than in Tower meal. Increased incubation time resulted in some decrease in total nitrile content in raw Tower and Midas RSM. Hydroxy nitriles (1-cyano-2-hydroxy-3-butene (N2) and isomeric 1-cyano--2-hydroxy-3,4-epithiobutanes (N4 and N5)) were less

Table III.9 Nitriles and goitrin content of treated rapeseed meals(mg/g meal)[1]

Variety	Heat Treatment	Method of Hydrolysis	Incubation Time (h)	Nitriles[5]					Total Nitriles	Goitrin
				N1	N2	N3	N4	N5		
Tower	Raw	Autolysis[2]	1	0.968	0.313	0.563	0.391	0.369	2.605	0.573
			12	0.668	0.332	0.360	0.402	0.385	2.147	0.624
			24	0.443	0.316	0.204	0.380	0.353	1.696	0.612
Tower	Raw	PHFD[3]	24	0	0.069	0	0.032	0.038	0.139	0.466
Tower	Heated	Autolysis	1	0	0.027	0	0	0	0.027	0.143
Tower	Heated	Hydrolysis[4]	24	0	0.033	0	0	0	0.033	1.648
Tower	Heated	PHFD	24	0	0.031	0	0	0	0.031	0.772
Midas	Raw	Autolysis	1	2.401	2.514	0.222	2.410	2.319	9.866	0.462
			12	1.851	2.327	0.210	2.084	2.063	8.535	0.425
			24	1.248	2.173	0.121	2.131	2.125	7.798	0.512
Midas	Raw	PHFD	24	0.267	2.626	0.036	1.083	1.312	5.324	2.662
Midas	Heated	Autolysis	1	0	0.090	0	0	0	0.090	0.155
Midas	Heated	Hydrolysis	24	0.007	0.306	0.008	0.008	0.012	0.341	10.095
Midas	Heated	PHFD	24	0.004	0.566	0.008	0.003	0.012	0.593	10.542

[1] Average value of duplicate samples; values for autolysis and hydrolysis samples are potential aglucon level while those for PHFD samples are actual aglucon level in meals.
[2] Hydrolyzed with endogenous enzyme.
[3] Pre-hydrolyzed with 10% ground raw rapeseed and freeze-dried.
[4] Hydrolyzed with 10% ground raw rapeseed.
[5] N1: 1-cyano-3,4-epithiobutane
N2: 1-cyano-2-hydroxy-3-butene
N3: 1-cyano-4,5-epithiopentane
N4: 1-cyano-2-hydroxy-3,4-epithiobutane (threo)
N5: 1-cyano-2-hydroxy-3,4-epithiobutane (erythro)

influenced by incubation time than non-hydroxy thionitriles (1-cyano-3,4-epithiobutane (N1) and 1-cyano-4,5--epithiopentane (N3)). Levels of goitrin, which are low in raw RSM and high in hydrolyzed heated RSM, were not significantly influenced by incubation time. Autolysis of heated RSMs showed the presence of very small amounts of 1-cyano-2-hydroxy-3-butene (N2) and goitrin.

Freeze-drying following hydrolysis influenced the nitrile and goitrin levels in RSMs. The PHFD sample of raw Tower seed did not contain any non-hydroxy thionitriles (N1, N3) and contained only one-tenth as much hydroxy thionitriles (N4, N5) and one-fifth as much 1-cyano--2-hydroxy-3-butene (N2) as found in raw Tower meal autolyzed for 24 h. The goitrin content of the meals was much less affected by freeze-drying in both PHFD samples of raw and heated Tower meals. The nitrile content of PHFD raw Midas meal was decreased by freeze-drying in a similar way to that of PHFD raw Tower meal except for the higher stability of 1-cyano-2-hydroxy-3-butene. Because the original samples of PHFD meals were lost, different batches of rapeseeds which had been stored for 6 months more than original samples were used to prepare a replacement for the original samples. The higher content of goitrin in PHFD raw Midas meal than in autolyzed raw Midas meal may have been related to the effect of time on the level of goitrin. A similar effect of aging on goitrin formation was observed in *Crambe* seed (VanEtten et al. 1969b, Tookey and Wolff 1970).

In general, goitrin showed much more stability than nitriles during incubation and freeze-drying. Among the nitriles 1-cyano-2-hydroxy-3-butene (N2) showed the greatest stability. This may have been related to hydrogen bonding between its hydroxyl group and other cell components such as polysaccharides. The isomeric 1-cyano-2-hydroxy--3,4-epithiobutanes may also have hydrogen bonding but the terminal thio-ring may have rendered them instable. The instabiity of isolated hydroxy epithionitriles from *Crambe* seed were observed by Daxenbichler et al. (1968) and VanEtten et al. (1969a). The lack of stability of the non-hydroxy thionitriles (N1, N3) during processing and incubation with changing pH was observed in an earlier study (Section A).

Analysis of PHFD raw Tower and Midas meal by GLC showed the presence of an unidentified compound as indicated by the peak (UK) occurring right after that of the isomeric hydroxy epithionitriles (Figure III.8). The UK peak was smaller than those of hydroxy epithionitriles before pre-hydrolysis and freeze-drying but became much larger after treatment. In a preliminary experiment a peak (X) which had similar retention time as the present UK peak appeared when the mixture of pure hydroxy epithionitriles (N4, N5) was stored in organic solvent (methylene chloride) for an extended period of time. Since the polymerization of N4 and N5 was indicated (Daxenbichler et al. 1968; VanEtten et al. 1969a) and the peak (X) appeared at the expense of N4 and N5, it

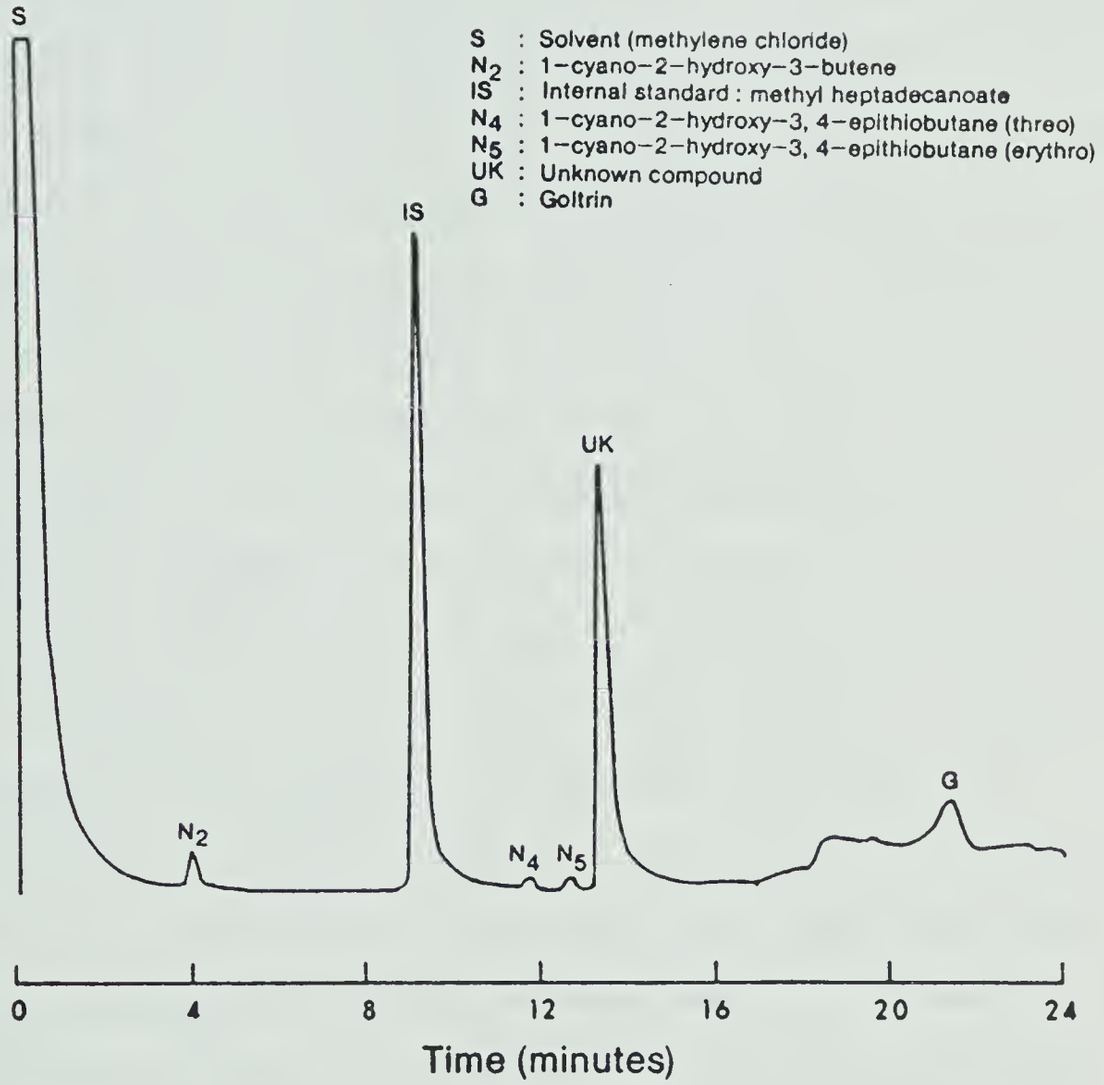


Figure III.8 Gas-liquid chromatography of hydrolysis products of pre-hydrolyzed, freeze-dried Tower meal

was presumed that the compound of the peak (X) was a degradation product of polymer of N4 and N5. Due to the similarity of retention time of compound UK and compound X an attempt was made to see if these compounds are of the same origin. Mass spectrum of the unknown (UK) (Appendix 1) showed fractions at 181, 180, 165, 137, 122, 94, 91, 79, 77, 66, 65, 53 and 51 m/e with base peak at 180 m/e. Possible molecular weight measured by high resolution MS was 180.0792 and the most likely formula is $C_{10}H_{12}O_3$. The IR spectrum (Appendix 2) showed the presence of the carbonyl group and absence of the functional group of nitrile. This compound (UK) could not be detected by iodine vapor on silica gel TLC and was unstable during fractionation by GLC (5% EGSS-X on Gas-Chrom Q). On the other hand the mass spectrum of compound X (Appendix 3) showed fractions at 149, 113, 85, 70, 69, 68, 58, 57, 44, 43, 42, 41, 40 m/e with base peak at 57 m/e. Despite a similar retention time of the compound UK to that of the compound X it appears that they are different. They have a different mass spectrum and the amount of UK produced by PHFD raw Midas meal was almost the same as produced by PHFD raw Tower meal. If UK was a degradation product of polymers of N4 and N5 one would expect greater production of UK from Midas meal. Identification of either precursor(s) or structure of the UK was not accomplished in this experiment.

Effects on the Performance of Broiler Chicken:

Examination of the data of broilers showed that weight

gain and feed intake of broilers fed 20% Tower RSMs, either raw or heated, was slightly less than that observed in birds fed the control ration (Table III.10). In addition, the method of hydrolysis used had little effect on weight gain or feed intake. No significant differences in feed efficiency were observed. Weight gain and feed intake of the birds fed 20% Midas RSMs were greatly reduced compared to the controls and those fed Tower RSMs. The effect was much more pronounced in the groups receiving the raw Midas meal than in those fed heated Midas meals. The method of hydrolysis treatment of the Midas meals had a pronounced effect on performances of birds fed treated meals. Inclusion of meals that were pre-hydrolyzed and freeze-dried resulted in decreased rate of growth and feed intake compared to the groups fed meals along with either 2% raw or heated ground rapeseed.

Inclusion of either Tower or Midas RSMs in the rations resulted in enlargement of the thyroid glands. The enlargement that occurred appeared to be directly related to levels of goitrin intake. In the groups fed PHFD meals, in which the levels of goitrin were known, calculation of the average daily intake of goitrin gave values for rations 1, 4, 7 and 10 of 33, 56, 72 and 514 moles per day respectively and the corresponding weights of their thyroid glands were 13.5, 21.7, 38.0 and 166.8 mg per 100 g body weight. Birds fed Tower meals had thyroid glands that were 2 to 3 times larger than in those fed the control ration.

Table III.10 Weight gain, feed intake, feed efficiency, thyroid glands weight, mortality and perosis of chicken fed experimental diets

Treatment Number	Variety	Heat Treatment	Method of Hydrolysis	Weight Gain (g/bird)	Feed Intake (g/bird)	Feed Efficiency (feed/gain)	Thyroid Glands wt. (mg/100 g body wt.)	Mortality (bird)	Perosis (bird)
1			PHFD[1]	676.8*ef*	1169.5*f*	1.73*b*	13.52*f*	0	2
2		Raw	R. Tower[2]	669.4*ef*	1155.8*f*	1.73*b*	21.37*def*	1	1
3			H. Tower[3]	632.8*e*	1112.8*def*	1.76*b*	19.30*ef*	1	0
	Tower								
4			PHFD	716.1*f*	1197.4*f*	1.68*b*	21.74*def*	0	0
5		Heated	R. Tower	653.2*e*	1169.4*f*	1.80*b*	17.12*ef*	1	0
6			H. Tower	665.0*ef*	1140.6*ef*	1.72*b*	18.34*ef*	1	0
7			PHFD	204.6*a*	444.4*a*	2.19*a*	37.95*cd*	0	0
8		Raw	R. Midas	312.0*b*	650.4*b*	2.09*a*	61.00*b*	0	0
9			H. Midas	288.7*b*	613.6*b*	2.13*a*	46.78*bc*	0	0
	Midas								
10			PHFD	455.6*c*	837.7*c*	1.85*b*	166.84*a*	1	0
11		Heated	R. Midas	586.3*de*	1037.3*d*	1.77*b*	48.48*bc*	3	1
12			H. Midas	572.4*d*	1056.8*de*	1.85*b*	33.55*cde*	1	0
13	Soybean meal control			717.3*f*	1297.6*g*	1.81*b*	7.73*f*	1	3
	SEM[4]			18.0	28.7	0.05	5.47		

[1] Pre-hydrolyzed and freeze-dried.
[2] 2% ground raw rapeseed was added to the diet.
[3] 2% ground heated rapeseed was added to the diet.
[4] Standard error of mean.
*a-g*Means in the same column followed by different letters differ ($p<0.05$).

Birds fed Midas meals had much larger thyroid glands, 4 to 20 times greater, than in the control group. By far the largest thyroid glands were noted in the groups fed PHFD heated Midas meal.

The overall results obtained reflected the levels of nitrile and goitrin in the meals when they were ingested and confirmed the earlier findings (VanEtten et al. 1969a; Srivastava et al. 1975) that a high level of nitrile present in autolyzed raw meals is more toxic than a high level of goitrin in heated meals hydrolyzed with myrosinase.

The reduced weight gain of chicks fed PHFD raw Tower meal as compared to those fed PHFD heated Tower meal indicates that even a small amount of nitriles and/or the unidentified compound (UK) may depress the growth of chicken; however, the differences noted were not significant. The greater body weight gain of chickens fed PHFD heated Tower meal (Tr. 4) than that of those fed heated Tower meal with raw ground Tower seed (Tr. 5) might be attributed to partial elimination of volatile hydrolysis products of glucosinolates and perhaps to slightly higher dry matter content in the PHFD meal. Addition of ground raw Tower rapeseed to heated Tower RSM (Tr. 6) had no effect on performance compared to heated Tower RSM which had no source of myrosinase (Tr. 5). The fact that PHFD raw Midas, which lost about 40% of nitriles during processing by either evaporation or polymerization, had a greater growth-depressing effect than raw Midas meal was indicative of the

differences in nitrile formation between *in vitro* and *in vivo* conditions. An earlier experiment (Section A) showed that the formation of nitriles in raw Midas meal was complete after 1 h of incubation at 37°C at a pH of approximately 5.5. The digestive tract of chicken has an average pH of 5.7 varying from 2.94 in the gizzard to 6.82 in the rectum (Schaible 1976). The lesser influence of raw Midas meals in comparison to PHFD raw Midas meal on weight gain indicated that the activity of factors that affect the formation of nitriles may have been suppressed in the gastro intestinal tract. The fact that the birds fed the heated Midas meal with ground raw Midas rapeseed (Tr. 11) were not affected as compared to those fed heated Midas meal without an external source of myrosinase (Tr. 12) and did not show as much thyroid enlargement as the PHFD heated meal (Tr. 10) suggests that inclusion of a source of myrosinase in the diet does not result in goitrin formation in the digestive tract in any way equivalent to that observed when the meal is pre-hydrolyzed *in vitro*. In a previous experiment (Section A), it was shown that addition of 5 to 10% (w/w) ground raw rapeseed to a heated meal resulted in maximum goitrin formation within 12 h. Since the response of thyroid glands weight to the intake of goitrin from PHFD meals appeared to be almost linear, it might be possible to estimate by extrapolation the levels of goitrin formed *in vivo* from other meals. The greater goitrogenic effect of heated Midas without a source of myrosinase (Tr. 12) than

Tower meals indicated that some hydrolysis of progoitrin occurred through myrosinase activity of bacteria in the gastro intestinal tract as reported by Greer (1962) and Oginsky et al. (1965).

Mortality was higher in the group fed heated Midas meal which is potentially high in goitrin content. In contrast, Marangos et al. (1974) observed that mortality was not related to the goitrin content of the rapeseed fed.

The incidence of perosis in the experiment was not affected by the treatments used. Inclusion of either raw or heated Tower or Midas meals apparently had no effect on the occurrence of leg abnormality even though others (Holmes and Roberts 1963; Ahlström et al. 1978) have reported increased incidence of perosis when RSMs of certain varieties were fed.

Summary

An experiment was conducted to study the effect of heat treatment and conditions of enzyme hydrolysis of Tower and Midas RSM on the concentration of nitriles and goitin in the meals and their biological effect when fed to broiler chickens. Autolysis of raw meals resulted in a high level of nitriles in Midas meal and a low level of nitriles in Tower meal. In heated meals, goitrin was the principal hydrolysis product being high in Midas and low in Tower meals. An increase in incubation time, and freeze-drying after hydrolysis reduced the total nitriles. Non-hydroxy epithionitriles were most susceptible to treatments imposed

and a hydroxy nitrile, 1-cyano-2-hydroxy-3-butene, was least affected by freeze-drying. Feeding 20% raw or heated Tower RSM hydrolyzed in different ways had little effect, compared to the soybean control, on rate of growth, feed efficiency, mortality or incidence of perosis. Thyroid gland weights in the treated groups were two to three times greater than the controls. Feeding 20% raw or heated Midas meal decreased rate of growth and increased feed required per unit of gain. The growth depression was greater with raw Midas RSM than with the heated meal. Thyroid gland weights in the groups fed Midas RSM, either raw or heated, were larger than for Tower RSM. Thyroid gland weights were 4 to 20 times as large as the controls. Raw or heated Midas RSM prepared by pre-hydrolysis and freeze-drying (PHFD) caused a greater decrease in rate of gain than other Midas meals in which 2% raw or heated ground Midas rapeseed was included in the rations fed.

C. The Effect of Sodium-thiosulfate and Hydroxo-cobalamin on Rats Fed Nitrile-rich or Goitrin-rich Rapeseed Meals.

Introduction

Cyanogenetic compounds are widely distributed in the plant kingdom and occur mainly in the form of cyanogenetic glucosides. Cyanogenetic glucosides such as amygdalin, dhurrin, linamarin and lotaustralin are glycosidic derivatives of α-hydroxynitriles (cyanohydrin). On enzymatic hydrolysis they yield glucose and α-hydroxynitriles which degrade, in most cases, to hydrogen cyanide (HCN) and the corresponding aldehyde or ketone (Conn 1973a,b; Montgomery 1969).

The glucosinolates present in species of *Crucirerae* and some other families also produce various nitriles under certain hydrolysis conditions but they are not α-hydroxynitriles. However, highly toxic effects of nitrile-rich preparations of *Crambe* seed meal (Tookey et al. 1965; VanEtten et al. 1969a) and RSM (Srivastava et al. 1975; Section B) have been observed. The β-hydroxynitriles produced from progoitrin (2-hydroxy-3-butenylglucosinolate) were about eight times as toxic as (R)-goitrin with mice as the test animal (Booth and Robbins 1968). Støa (1952) suggested the following detoxification scheme in which R is an aliphatic or aromatic group;

$$R\text{–}CH_2CN \rightarrow R\text{–}CHOHCN \rightleftharpoons \begin{matrix} R\text{–}CHOH \\ \downarrow \\ R\text{–}COOH \end{matrix} + \begin{matrix} HCN \\ \downarrow \\ HSCN \end{matrix}$$

Aliphatic or aromatic nitriles are first converted to

α-hydroxynitriles *in vivo* and then they are degraded into aldehyde and hydrogen cyanide which forms thiocyanate to be excreted. The velocity of the reaction, which splits off HCN, is the main factor determining the toxicity of cyano compounds and obviously the oxidative reactivity of the α-carbon atom plays a primary role. Ghiringhelli (1956) reported that the toxicity of the nitriles was independent of their molecular weight but probably dependent upon their water solubility. In general, saturated aliphatic and aromatic nitriles are no more toxic than many chemicals commonly regarded as relatively harmless. Unsaturated nitriles, cyanohydrins and α-aminonitriles, however, approach hydrogen cyanide in toxicity (Mowry 1948).

In cyanide poisoning, a safe level of methemoglobinemia was induced by administration of *p*-aminopropiophenone or sodium nitrite therapeutically and prophylactically (Rose et al. 1947; Yoshikawa 1968). Methemoglobin competes with cytochrome c oxidase for cyanide ions (Albaum et al. 1946). In combination with methemoglobinemia inducing drugs, other compounds have also been shown to exert some effect on the toxicity of cyanide. Sodium thiosulfate was used to react with cyanide ion to form thiocyanate which is much less toxic (Rose et al. 1947). Hydroxo-cobalamin (vitamin B_{12}a) has also been shown to exert some antidotal efficacy in experimental cyanide poisoning. Administration of hydroxo--cobalamin resulted in a comparatively large quantity of cyano-cobalamin (vitamin B_{12}) being excreted in the urine of

mice treated with hydroxo-cobalamin and potassium cyanide but the precise site where this reaction had taken place was not known (Mushett et al. 1952).

Since sodium thiosulfate and hydroxo-cobalamin have been shown to partially reduce the toxicity of the cyanide ion which could be produced from some nitriles, it seemed desirable to determine whether these compounds might reduce the toxicity of nitriles in RSM. Consequently, three experiments were conducted to study the biological effects of dietary supplementation of sodium thiosulfate and hydroxo-cobalamin on rats which were fed RSMs prepared to contain high levels of nitriles or goitrin.

Experimental

Rapeseed meals with varying levels of nitriles and goitrin were prepared according to the method used previously (Section B). The levels of nitriles and goitrin in the RSM preparations were determined by the GLC method used previously.

Three experiments each of 4 weeks duration were conducted using weanling male Wistar rats. Rats for each group were selected to give groups of similar average body weight. The rats were assigned at random to individual cages. Feed and water were supplied *ad libitum*. Weekly weight gain was recorded and feed consumption and organ weight were measured when the experiments were terminated. The thiocyanate ion content of serum and urine was determined by the method of Bowler (1944) and background

interference was adjusted by using mercuric chloride (Johnston and Jones 1966).

Data obtained were analyzed by the same statistical method used in Section B.

Experiment 1:

Rapeseeds of two *B. napus* varieties, Tower and Midas, were used to prepare meals with varying levels of nitriles and goitrin. Either raw or heated seeds of each variety were ground, extracted with hexane, pre-hydrolyzed with ground raw seed and freeze-dried (PHFD). The effect of the addition of two levels of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) (0.1 and 0.3%) to the rations with Tower meals and one level of either sodium thiosulfate (0.1%) or one level of hydroxo--cobalamin (0.1%) to the rations with Midas meals were compared to rations containing rapeseed meals without any additives. Forty-eight weanling rats weighing 76.4 g on average were selected and 4 rats were assigned to each treatment.At the end of the experiment, the thyroid glands and kidneys were removed and their weights were recorded.

Experiment 2:

Meals of potentially high nitrile or high goitrin content were prepared using either raw or heated Midas seed. The seed was ground and extracted with hexane but was not pre-hydrolyzed. The experimental diets were similar to the one used in Experiments 1 and 3 (Table III.11) but instead of 20% PHFD meal 18.2% of Midas meal and 1.8% of ground Midas rapeseed were added. With the groups fed raw Midas

Table III.11 Composition of diets of rats

Ingredients	Experiments	
	1 & 3	2
	%	%
Ground wheat	60.8	60.8
Soybean meal	10.0	10.0
PHFD rapeseed meal[1]	20.0	0
Rapeseed meal	0	18.2
Ground rapeseed	0	1.8
Stabilized animal fat	7.0	7.0
Ca-phosphate	0.5	0.5
Limestone	1.0	1.0
Iodized salt	0.2	0.2
Micromix[2]	0.5	0.5
Calculated composition:		
Metabolizable energy (MJ/kg)	13.81	
Crude protein (%)	20.4	
Ca (%)	0.6	
P (%)	0.6	

[1] Pre-hydrolyzed and freeze-dried rapeseed meal.
[2] Supplied the following levels per kg diet:
Mn 63 mg; Zn 40 mg; Se 0.05 mg; Vitamin A 10,000 IU; Vitamin D_3 1,000 IU; Vitamin E (α) 35 mg; Vitamin K 0.05 mg; Choline chloride 750 mg; Niacin 15 mg; Ca-pantothenate 8 mg; Riboflavin 2.5 mg; Vitamin B_{12} 0.005 mg; Biotin 0.1 mg.

meal the effects of adding 0.1% sodium thiosulfate or 0.1% hydroxo-cobalamin were compared to those without additives. In the groups fed heated Midas meals the effect of adding either 0.02% or 0.1% hydroxo-cobalamin were compared to a group without additive. Organ weights and thiocyanate excretion of the treated groups were compared to those of control rats fed a commercial ration. Seventy weanling rats averaging 86 g each were selected and 10 rats were assigned to each group. After 28 days on experiment, 3 rats from each group were placed in metabolic cages and urine output was collected for two days. The urine from each rat was analyzed for thiocyanate content. At the end of the experiment the rats were killed and their thyroid glands and kidneys were removed and weighed.

Experiment 3:

Experiment 3 was conducted to repeat treatments which had caused changes in performance in Experiment 1, namely the addition of sodium thiosulfate to PHFD raw Midas meal and the addition of hydroxo-cobalamin to PHFD heated Midas meal. The treatments used are summarized in Table III.15. Forty five weanling rats averaging 64 g in weight were divided into 5 comparable groups of 9 rats each and placed on the different treatments. Five samples of urine for thiocyanate determination were obtained from each group. Blood samples were obtained by cardiac puncture. At the end of the experiment the kidneys, liver and thyroid glands from each rat were removed and weighed. The color of liver was

visually examined and changes were recorded.

Results

The levels of nitriles and goitrin in the rapeseed meals used in each experiment are shown in Table III.12. The patterns of aglucone products in treated RSMs were similar to those shown in Section B. The PHFD raw Midas meal used in Experiment 1 contained more nitriles and less goitrin than the meal used in Experiment 3. When raw Midas meal was autolyzed the concentration of nitriles was high and when heated Midas meal was hydrolyzed with 1% mustard myrosinase the level of goitrin was high (Experiment 2).

Experiment 1:

The results obtained from Experiment 1 are summarized in Table III.13 and weekly cumulative weight gain curves of selected groups are shown in Figure III.9. Rats fed PHFD Tower meal gained significantly more weight than those fed PHFD Midas meal. Heat-treatment of the seeds of both varieties resulted in increased weight gain of rats when the meals were fed. The addition of 0.1% sodium thiosulfate to PHFD raw Midas meal and PHFD raw Tower meal resulted in increased weight gain but the increase only reached statistical significance with the Midas meal which had a high nitrile content. The addition of 0.1% sodium thiosulfate to the raw Midas meal also resulted in a significant improvement in feed conversion. Addition of 0.3% sodium thiosulfate to either PHFD raw or heated Tower meals had no significant effect on weight gain or feed efficiency.

Table III.12 Nitriles and goitrin content of rapeseed meals used in rat experiments (mg/g meal)[1]

Exp't	Variety	Heat Treatment	Method of Hydrolysis	Nitriles[5]					Total Nitriles	Goitrin
				N1	N2	N3	N4	N5		
1	Tower	Raw	PHFD[2]	0	0.069	0	0.032	0.038	0.139	0.466
		Heated	PHFD	0	0.031	0	0	0	0.031	0.772
	Midas	Raw	PHFD	0.267	2.626	0.036	1.083	1.312	5.324	2.662
		Heated	PHFD	0.004	0.566	0.008	0.003	0.012	0.593	10.542
2	Midas	Raw	Autolysis[3]	1.563	2.483	0.270	1.574	1.600	7.490	3.731
		Heated	Hydrolysis[4]	0	0.496	0.030	0	0	0.526	10.598
3	Midas	Raw	PHFD	0.208	2.035	0.028	0.660	0.870	3.802	3.791
		Heated	PHFD	0.001	2.356	0	0.005	0.010	0.372	11.894

[1] Average value of duplicate samples.
[2] Pre-hydrolyzed with 10% (w/w) ground raw rapeseed and freeze-dried.
[3] Hydrolyzed with endogenous enzyme for 1 h at 37°C for analysis of potential aglucon products.
[4] Hydrolyzed with 1% (w/w) mustard myrosinase for 1 h at 37°C for analysis of potential aglucon products.
[5] N1: 1-cyano-3,4-epithiobutane
N2: 1-cyano-2-hydroxy-3-butene
N3: 1-cyano-4,5-epithiopentane
N4: 1-cyano-2-hydroxy-3,4-epithiobutane (threo)
N5: 1-cyano-2-hydroxy-3,4-epithiobutane (erythro)

Table III.13 The performance and organ weight of rats fed PHFD rapeseed meals supplemented with sodium thiosulfate or hydroxo-cobalamin (Experiment 1)

Variety	Heat Treatment	Additives	Weight Gain (g)	Feed Intake (g)	Feed Efficiency (feed/gain)	Thyroid Weight (mg/100 g body wt.)	Kidney Weight (mg/100 g body wt.)
Tower	Raw	None	199.4*d*	501.0*cd*	2.52*a*	9.5*ab*	842*bc*
		0.1% S.T.[1]	215.5*de*	518.8*cd*	2.41*a*	8.3*a*	827*abc*
		0.3% S.T.	198.1*d*	483.5*c*	2.45*a*	10.1*abc*	869*c*
	Heated	None	218.5*e*	536.5*d*	2.46*a*	11.4*abc*	822*abc*
		0.1% S.T.	220.8*e*	527.0*cd*	2.39*a*	10.9*abc*	867*c*
		0.3% S.T.	211.6*de*	515.5*cd*	2.44*a*	12.7*bc*	811*abc*
Midas	Raw	None	48.2*a*	209.5*a*	4.35*c*	11.6*abc*	1111*e*
		0.1% S.T.	83.2*b*	266.8*b*	3.21*b*	13.5*c*	1012*d*
		0.1% H.C.[2]	39.0*a*	198.3*a*	5.13*d*	13.4*c*	1218*f*
	Heated	None	98.0*bc*	298.8*b*	3.05*b*	22.5*d*	788*ab*
		0.1% S.T.	98.8*bc*	301.3*b*	3.05*b*	19.8*d*	778*a*
		0.1% H.C.	104.4*c*	309.5*b*	2.97*b*	21.6*d*	813*abc*
SEM[3]			5.9	13.9	0.08	1.2	18

[1] Sodium thiosulfate pentahydrate
[2] Hydroxo-cobalamin
[3] Standard error of mean
*a-f*Means in the same column followed by different letters differ ($P<0.05$).

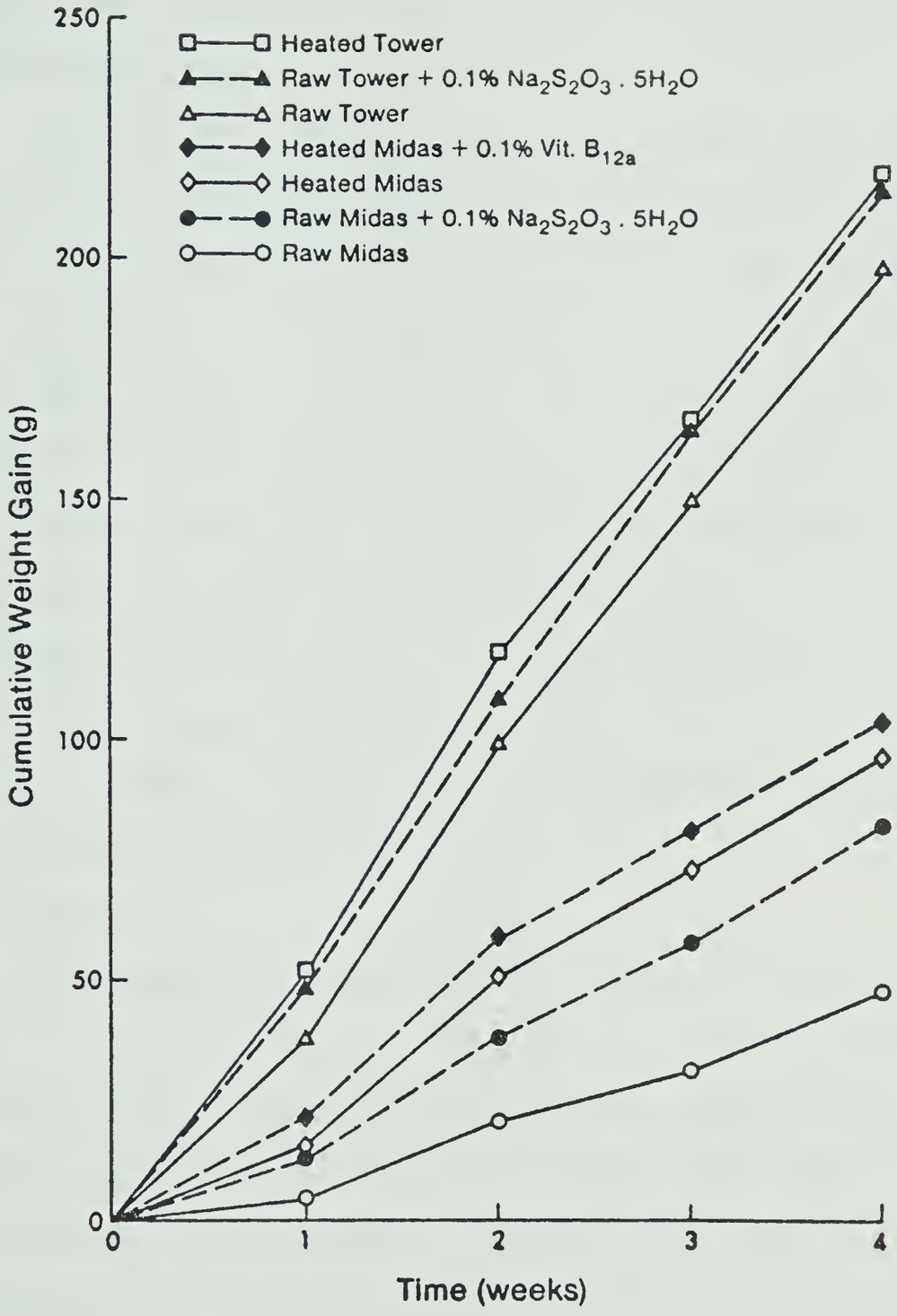


Figure III.9 Weight gain of rats fed pre-hydrolyzed freeze-dried rapeseed meal (Experiment 1)

The addition of 0.1% hydroxo-cobalamin to PHFD raw or heated Midas meals had no significant effect on performance although better weight gain was shown during the first week of experiment when hydroxo-cobalamin was added to the PHFD heated Midas meal.

The size of thyroid glands and kidneys was affected by some of the treatments used. Rats fed the PHFD heated Midas meal, which was high in goitrin content, had thyroids that were significantly heavier than those on other treatments. The kidney weight of rats fed PHFD raw Midas meals were significantly heavier than those of other groups. The addition of sodium thiosulfate or hydroxo-cobalamin did not influence thyroid or kidney weights except in the treatments involving raw Midas meals. In these treatments the addition of 0.1% sodium thiosulfate caused a decrease in kidney weight while the addition of 0.1% hydroxo-cobalamin resulted in increased kidney weight.

Experiment 2:

The results obtained in Experiment 2 are summarized in Table III.14. The groups fed rations containing heat-treated Midas meal with ground raw Midas seed added gained more weight and had better feed efficiency than those fed raw Midas meal. The addition of sodium thiosulfate or hydroxo--cobalamin did not influence weight gain or feed efficiency.

The thyroid glands of the groups fed either raw or heated Midas meal were significantly heavier than those of the controls. The weight of thyroid glands of the groups fed

Table III.14 The performance, organ weight and thiocyanate excretion of rats fed Midas rapeseed meals supplemented with sodium thiosulfate or hydroxo-cobalamin (Experiment 2)

Heat Treatment	Additives	Weight Gain (g)	Feed Intake (g)	Feed Efficiency (feed/gain)	Thyroid Weight (mg/100 g body wt.)	Kidney Weight (mg/100 g body wt.)	SCN^- Excretion (µg/day/ 100 g body wt.)
Raw	None	75.4*a*	285.6*a*	3.85*c*	24.2*de*	1009*c*	1882*bc*
	0.1% S.T.[1]	76.1*a*	285.6*a*	3.77*bc*	20.4*bcd*	986*c*	1810*bc*
	0.1% H.C.[2]	73.6*a*	290.6*a*	3.98*c*	26.8*e*	968*c*	1945*c*
Heated	None	103.8*b*	337.7*b*	3.28*a*	17.7*b*	841*a*	1443*b*
	0.02% H.C.	95.9*b*	332.8*b*	3.48*ab*	22.0*cd*	914*b*	1509*bc*
	0.1% H.C.	104.9*b*	338.8*b*	3.26*a*	17.9*bc*	909*b*	1515*bc*
Control		-	-	-	6.4*a*	898*b*	52*a*
SEM[3]		3.4	6.6	0.11	1.4	16	134

[1] Sodium thiosulfate pentahydrate
[2] Hydroxo-cobalamin
[3] Standard error of mean
*a-e*Means in the same column followed by different letters differ ($P<0.05$).

raw Midas meal were heavier than those fed heated meal. The kidney weights of rats fed raw Midas meal were heavier than those of rats fed heated Midas meal or the control ration. The inclusion of either raw or heated Midas meals in the diet resulted in a marked increase in excretion of thiocyanate in the urine. Levels of excretion were approximately 30 times greater than in the control group. The level of excretion was higher in rats fed raw Midas RSM than those fed heated meal.

Experiment 3:

The results obtained from Experiment 3 are summarized in Table III.15. The rats fed PHFD heated Midas meal gained more weight and consumed more feed than those fed PHFD raw Midas meal. Thyroid glands were heavier and kidneys were lighter for rats fed PHFD heated Midas meal compared with those receiving the raw meals. These results were similar to those obtained in Experiment 1.

The addition of sodium thiosulfate to the diet with PHFD raw Midas meal resulted in increased weight gain, and improved feed efficiency. Thyroid gland weight was not affected but kidney and liver weights, expressed on the basis of body weight, were reduced. Livers were lighter in color than in the rats fed PHFD raw Midas meal without sodium thiosulfate in which livers had a dark and greenish tinge. The addition of sodium thiosulfate resulted in increased thiocyanate levels in serum and reduced levels in the urine.

Table III.15 The performance, organ weight, and thiocyanate content in serum and urine of rats fed PHFD Midas meals supplemented with sodium thiosulfate or hydroxo-cobalamin (Experiment 3)

Heat Treatment	Additives	Weight Gain (g)	Feed Intake (g)	Feed Efficiency (feed/gain)	Thyroid Weight (mg/100 g body wt.)	Kidney Weight (mg/100 g body wt.)	Liver Weight (g/100 g body wt.)	SCN^- Serum (µg/1 ml)	SCN^- Urine (µg/day/ 100 g body wt.)
Raw	None	51.0*a*	180.0*a*	3.71*b*	18.0*b*	1337*c*	7.71*d*	6.70*b*	460*c*
	0.1% S.T.[1]	65.0*b*	206.8*b*	3.21*a*	18.9*b*	1200*b*	6.72*c*	8.84*c*	373*b*
Heated	None	82.5*c*	267.3*c*	3.27*ab*	32.7*c*	849*a*	6.22*bc*	6.12*b*	400*bc*
	0.1% H.C.[2]	81.6*c*	263.4*c*	3.27*ab*	27.8*c*	856*a*	6.02*b*	6.45*b*	355*b*
Control		-	-	-	7.6*a*	922*a*	5.01*a*	3.78*a*	78*a*
SEM[3]		4.1	6.9	0.16	2.0	23	0.19	0.45	25

[1] Sodium thiosulfate pentahydrate
[2] Hydroxo-cobalamin
[3] Standard error of mean
*a-d*Means in the same column followed by different letters differ ($P<0.05$).

In this experiment the addition of 0.1% hydroxo--cobalamin to a ration containing PHFD heated Midas meal had no effect on any of the parameters measured.

The liver weights of all groups fed PHFD raw or heated Midas meal were heavier than those of the control. The livers of rats fed PHFD raw Midas meal were heavier and darker in color than those of heated meal. The rats fed the control ration had smaller thyroid glands and liver than any of the treatment groups. Levels of thiocyanate in serum and urine of the control were much lower than those fed either raw or heated Midas RSM.

Discussion

Levels of nitriles and goitrin in different preparations of rapeseed meals may be quite variable. The nitrile content of the raw meals used in these experiments was consistently lower and the goitrin content was higher than those observed in previous studies (Section A and B). There was also some variability in levels of nitriles and goitrin in the Midas meals used in this study (Experiments 1 and 3). The meals were prepared at different times and therefore storage period may have affected the content of aglucone products as was observed by others in older seed (VanEtten et al. 1969b; Tookey and Wolff 1970).

Improved performance from heat treatment of Midas meal may be attributed to the prevention of nitrile formation in PHFD meal and to the fact that nitrile is much more toxic than goitrin as was reviewed previously. The observation

that heat treatment of Tower rapeseed improved weight gain slightly ($p<0.1$) in rats differed from results obtained in Section B with chickens in which no improvement was observed. It is not clear whether the small amount of nitriles present in PHFD raw Tower meal was the reason for the difference in weight gain of rats. Species differences in nitrile toxicity have been observed between rabbits and chickens (Spence 1933) and between rats and chickens (Srivastava et al. 1975).

The beneficial effect of sodium thiosulfate in the high nitrile treatments of Experiments 1 and 3 indicates that ingested nitriles produce the cyanide group (CN) probably *via* the scheme suggested by Støa (1952) and that CN is detoxified to thiocyanate by sodium thiosulfate. Since the addition of hydroxo-cobalamin had no significant effect on growth of rats fed either raw meal or heated meal it does not appear that the hydroxo-cobalamin was involved in CN detoxification. Failure to observe an antidotal effect of hydroxo-cobalamin in contrast to the beneficial effect on cyanide poisoning reported by Mushett et al. (1952) may be due to the different method of administration (intravenous vs. oral).

When raw Midas meal was fed to rats without hydrolysis (Experiment 2) growth was not suppressed as much as when PHFD raw Midas meal was fed (Experiments 1 and 3) even though intake of potential nitrile-forming compounds was higher. This is similar to the results obtained in other

experiments in which chickens (Section B) or chickens and rats (Srivastava et al. 1975) were used. Auld (1912) reported that under digestive conditions cyanogenesis from cyanogenic glucosides were inhibited by acids and alkali, digestive juices, cellulose and glucose. Such inhibitory mechanisms could have influenced levels of nitriles formed from glucosinolates when non-hydrolyzed RSM was fed.

Effect of treatments on thyroid weight indicated that the size of the gland was generally influenced by the goitrin content of the meals, but the effects were not as marked as in a previous experiment with chickens. In Experiment 2, the rats fed heated Midas meal showed less thyroid gland enlargement than those fed raw Midas meal. This suggests that goitrin production may have been inhibited *in vivo* in the same way as the inhibition of nitrile may have occured. Although the addition of hydroxo--cobalamin tended to increase thyroid gland weight in Experiment 2, the overall effect of this additive on the weight of thyroid gland was not clear.

Rats fed meals of high nitrile content had kidneys that were significantly larger than those fed meals of high goitrin content or those fed a control ration without RSM. It was also noted that the liver weight of the high-nitrile group was heavier than that of the high-goitrin group. According to Israels et al. (1979), a large number of foreign compounds are metabolized by the mixed function oxidases of the hepatic endoplasmic reticulum and induction

by xenobiotics is associated with an increase in liver size. Enlarged kidney and liver of rats fed nitrile-rich meal have been reported by other authors (VanEtten et al. 1969a; Srivastava et al. 1975).

The significantly higher thiocyanate content in serum of rats fed PHFD raw Midas meal with sodium thiosulfate suggests that more rapid or greater detoxification of CN took place in this treatment. The excretion of greater amounts of thiocyanate in urine of rats fed raw Midas meal of high nitrile content than in heated meal (Experiments 2 and 3) is in accord with the observations of Srivastava and Hill (1975) when Zephyr RSM was fed. A comparison of Experiments 2 and 3 indicates that the level of thiocyanate excretion in Experiment 3 reflects the lower levels of nitriles in the PHFD raw Midas meal as compared with the potential level of nitrile in Experiment 2. Other thiocyanogenic factors, however, may have influenced the level of excretion. Since the thiocyanogenic property of isothiocyanate *in vivo* (Langer 1964) and the presence of thiocyanate ion in hydrolyzed RSM (Srivastava and Hill 1975; McGregor 1978) have been reported, thiocyanogenic compounds removed by PHFD must have been mainly volatile isothiocyanate, thiocyanate or volatile nitriles. The amount of thiocyanate excreted in the urine of rats fed PHFD meal was approximately 5 times that excreted by the control group. The fact that the high goitrin group excreted almost the same level of thiocyanate as the high nitrile group in

Experiment 3 is noteworthy. Jirousek (1956) reported that the goitrogenic substance methyl-thiouracil suppressed high thiocyanate excretion induced by continuous administration of thyroxin and thyroglobulin. Remnants of aglucone products and/or unknown thiocyanogenic compound(s) may have caused the increased level of thiocyanate excretion in Experiment 3. It is not clear why less thiocyanate was excreted when sodium thiosulfate was added than when none was included in the ration. The overall results suggest that, among the aglucone products of RSM, at least nitriles may produce CN which would be excreted in the urine in the form of thiocyanate.

Summary

Three experiments were conducted with rats to study the effects of feeding rapeseed preparations supplemented with sodium thiosulfate($Na_2S_2O_3 \cdot 5H_2O$) or hydroxo-cobalamin (vitamin B_{12}a). A significant antidotal effect of 0.1% sodium thiosulfate supplementation was observed with pre-hydrolyzed, freeze-dried (PHFD) raw Midas meal which is rich in nitrile content, but not with Midas meal which was not hydrolyzed. The antidotal effect of sodium thiosulfate was less conspicuous with Tower meal but addition of 0.1% sodium thiosulfate to PHFD raw Tower meal resulted in slightly improved performance. Addition of sodium thiosulfate to heated Midas or heated Tower meal had no effect on weight gain whether they were pre-hydrolyzed or not. No antidotal effect of hydroxo-cobalamin was observed

with meals of high-nitrile or high-goitrin content. The addition of sodium thiosulfate or hydroxo-cobalamin had no significant effect on the weight of thyroid glands. Increased kidney weight was observed in treatments in which meal of high nitrile content (raw Midas meals) was fed. The addition of hydroxo-cobalamin resulted in an increase in kidney weight in some treatments while the addition of sodium thiosulfate caused a decrease in kidney and liver weight in the high-nitrile treatments. A significantly higher thiocyanate content was observed in the serum of rats fed high-nitrile meals supplemented with sodium thiosulfate. Thiocyanate excretion in the urine was reduced when Midas meals were pre-hydrolyzed and freeze-dried but remained much higher than for the control group.

IV. GENERAL DISCUSSION

The experiments reported herein indicated that concentration of the hydrolysis products of glucosinolates in a number of rapeseed varieties grown in Western Canada was variable depending on the hydrolysis conditions and quantity of glucosinolates originally present in the seeds. Two non-hydroxy epithionitriles that had not been reported before in rapeseed meals were identified. They were among the autolysis products of the glucosinolates of raw RSMs prepared in the laboratory. The highest concentrations of the non-hydroxy epithionitriles were found in Torch RSM with the next highest concentration in Midas meal.

The results obtained clearly indicate that heating Midas rapeseed improved weight gain and feed efficiency of broilers although no improvement was noted when Tower rapeseed was heated. The performance of birds fed the raw and heated PHFD Midas meals supports the view that nitriles are more deleterious to the chicken than goitrin. Mixing 2% of ground raw rapeseed (equivalent to 10% of the RSM) in the diet did not affect performance of birds indicating that moderate contamination by raw seeds as a possible source of active myrosinase should not cause practical problems.

The antidotal effect of sodium thiosulfate when high-nitrile RSM was fed indicates that the cyanide group may be formed when raw rapeseed products are ingested. The use of sodium thiosulfate with diets containing rapeseed products derived from seeds that had been damaged and exposed to

heavy rains during the harvest season may have practical significance since nitriles might be formed in such seed. Studies with other known antidotes for cyanide poisoning, such as sodium nitrite, may also be desirable.

The high level of thiocyanate excretion by rats fed RSMs along with a source of myrosinase is of interest. It has been reported that following hydrolysis with myrosinase low-glucosinolate RSM contains as much thiocyanate ion as high-glucosinolate RSM but the exact precursors of thiocyanate ion in RSMs are not known (McGregor 1978). Since thiocyanate is one of the known goitrogens, further information on the influence of RSM on thiocyanate metabolism is required.

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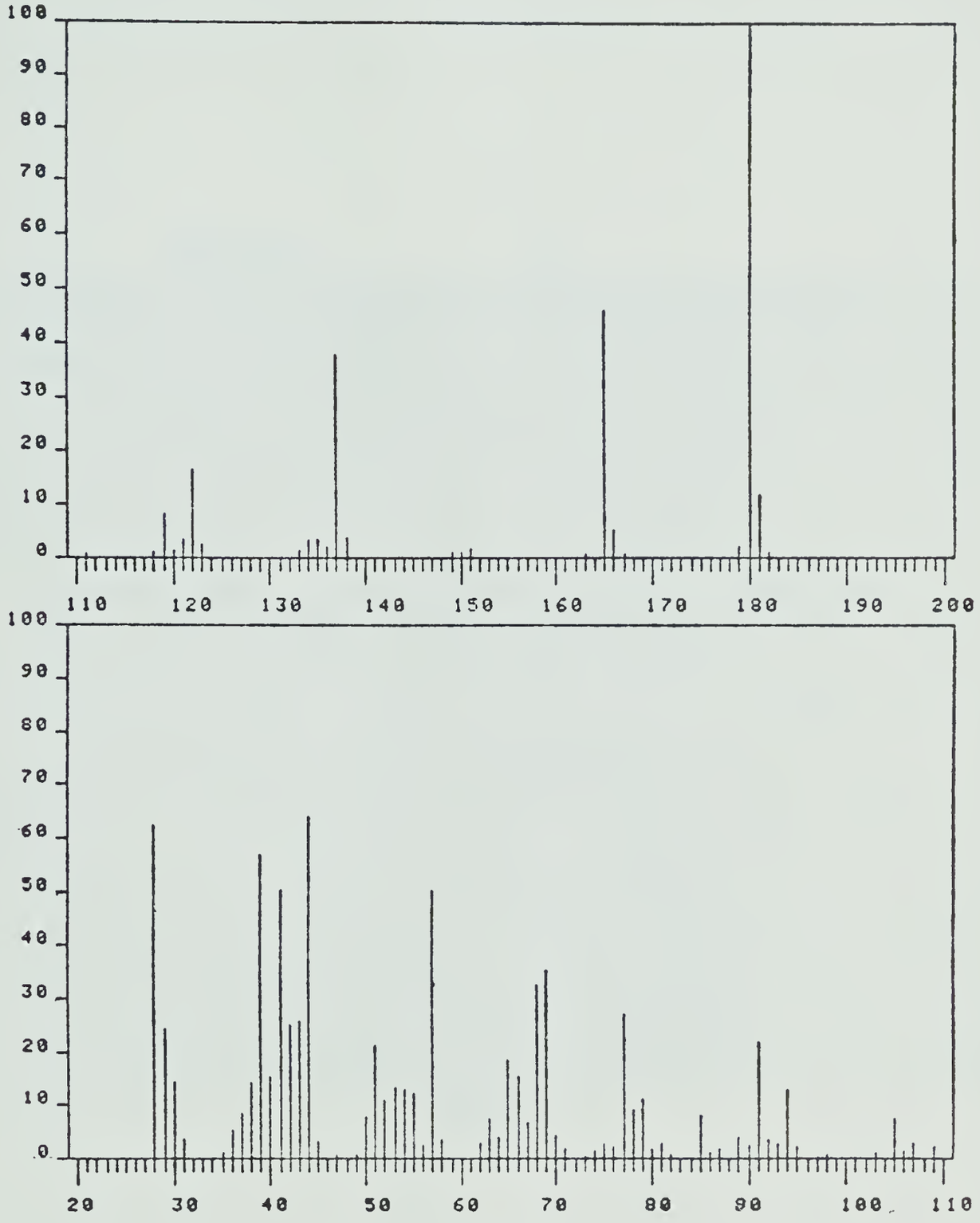
APPENDICES

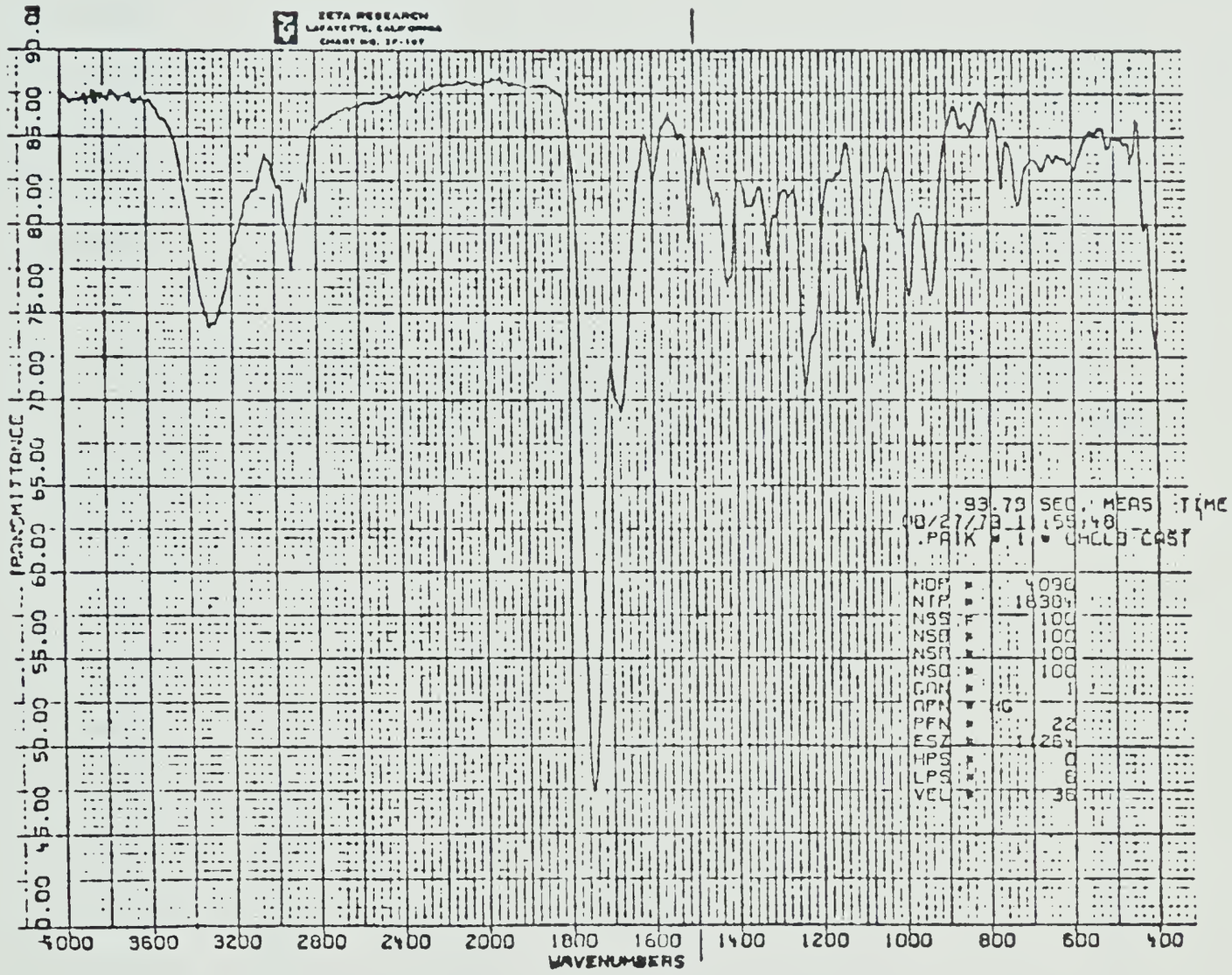
DS-50S MASS INTENSITY REPORT,

INKEE PAIK 1B DISK GH

N112.1 [TIC=25557, 100%=2475] EI



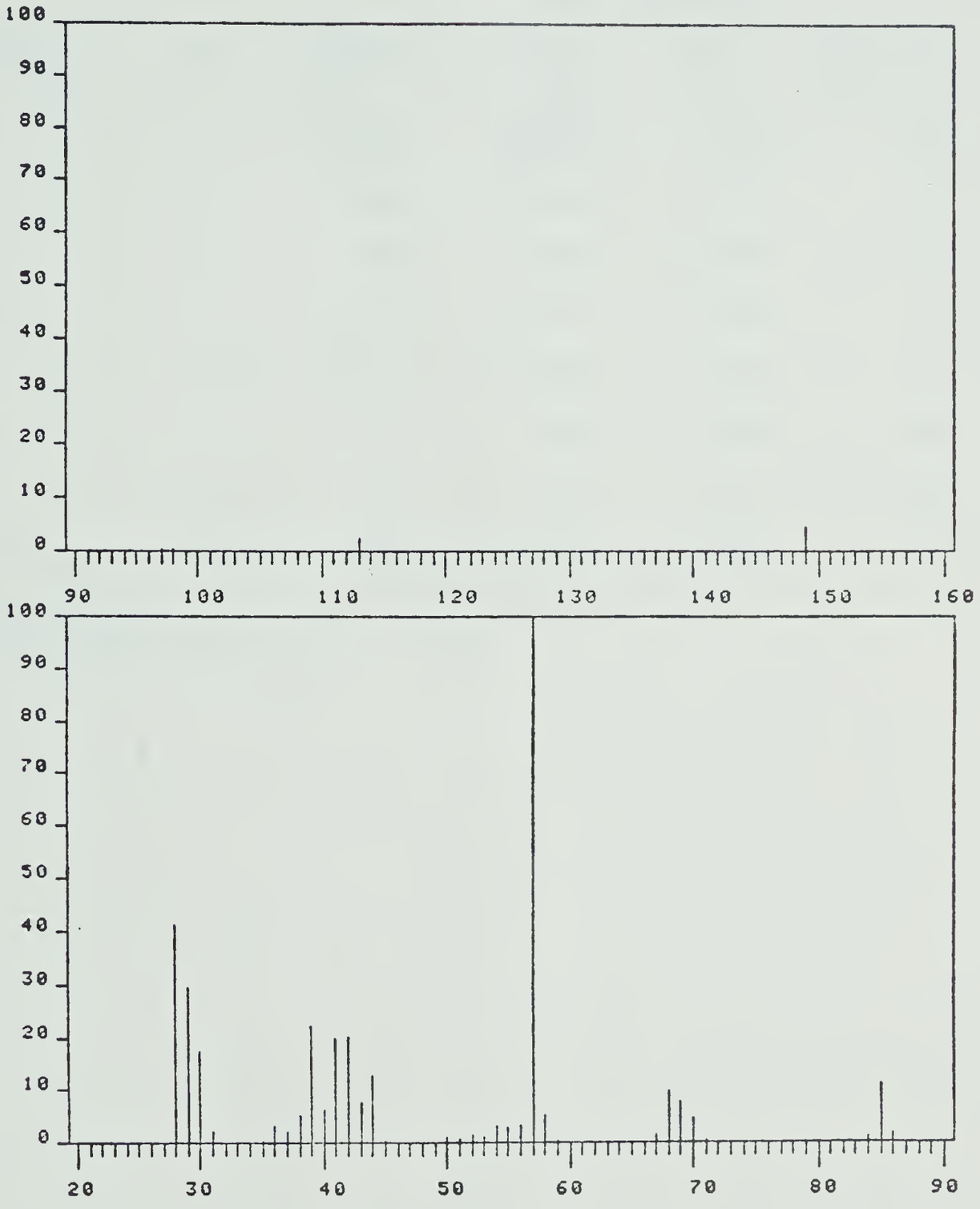
Appendix 1. Mass spectrum of compound UK



Appendix 2. IR spectrum of compound UK

DS-50 MASS INTENSITY REPORT: N144.144 [TIC=24557, 100%=6915]

INKEE PAIK STD. MIX. DISK S



Appendix 3. Mass spectrum of compound X

Appendix 4. Analysis of variance-source, degree of freedom (df) and mean squares-Broiler Experiment (Section B)

Source	df	Mean Squares			
		Weight Gain	Feed Intake	Feed Effic.	Thyroid Gland wt.
A	1	423207	885696	0.3602	13368
B	1	124502	278620	0.1504	1878
C	2	3592	17981	0.0007	2023
AB	1	94702	221971	0.1380	1670
AC	2	15228	37921	0.0102	2216
BC	2	417	657	0.0023	3881
ABC	2	778	364	0.0031	2858
Error	12	678	1711	0.0051	65

A: variety
B: heat-treatment
C: method of hydrolysis

Appendix 5. Analysis of variance-source, degree of freedom (df) and mean squares-Rat Experiments (Section C)

Experiment	Source	df	Mean Squares							
			Weight Gain	Feed Intake	Feed Effic.	Thyroid Gland wt.	Kidney wt.	Liver wt.	SCN^- Serum	SCN^- Urine
1-Tower	H	1	956	3825	5.42E-3	34.32	1001			
	A	2	369	1245	1.54E-3	6.40	437			
	HA	2	97	440	1.62E-3	0.44	4946			
	Error	18	220	1067	5.49E-3	2.71	824			
1-Midas	H	1	10790	34901	8.216	407.75	583662			
	A	2	838	2166	1.570	1.35	27248			
	HA	2	1179	2829	1.846	9.73	13448			
	Error	17	49	451	0.044	8.46	1760			
2	H	1	10533	3624231	4.170	315.1	1.49E-1			683670
	A/H	4	129	9263	0.134	80.8	1.04E-2			9262
	Error	54	118	43975	0.124	20.4	2.67E-3			-
	Error	12								62109
3	H	1	5225	46656	0.328	1257.9	1556962	10.696	20.01	7473
	A/H	2	443	1647	0.545	54.7	42441	2.295	10.56	12080
	Error	32	153	424	0.217	40.1	5232	0.362	1.98	3665

H: heat-treatment
A: additives